Regulation of the *C. elegans* RAS/MAPK Pathway by the Tumor Suppressor PTEN DAF-18 and Nutritional Cues

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Zusammenfassung

Multizelluläre Organismen befinden sich in ständiger Interaktion mit ihrer Umgebung, indem sie Umweltveränderungen wahrnehmen und entsprechend darauf reagieren. Eine Reaktion auf wechselnde Umweltbedingungen ist auf verschiedenen Ebenen ersichtlich; zum Beispiel auf der Ebene des ganzen Organismus, wenn sich ein Mensch bei Hitze in den Schatten begibt, auf der Ebene der Organe, wenn die Herzfrequenz bei körperlicher Anstrengung steigt, oder auf der Ebene einzelner Zellen, welche die Apoptose (den Zelltod) infolge ionisierender Bestrahlung auslösen.

Auch der Fadenwurm *C. elegans* reagiert in vielfältiger Art und Weise auf veränderte Umweltbedingungen. *C. elegans* sucht ständig nach den besten Bedingungen um sich optimal vermehren zu können. Wenn die Nahrung einen ausreichenden Nährwert hat, beendet *C. elegans* die Futtersuche und beginnt zu fressen. Weist die Nahrung aber nur einen geringen Nährwert auf, so setzt *C. elegans* seine Suche nach Futter fort, und sein Schlund (Pharynx) pumpt kontinuierlich, um so viel Nahrung wie möglich aufzunehmen. Ist gar keine Nahrung vorhanden, so entwickelt sich die *C. elegans* Larve in eine langlebige, so genannte Dauerlarve. Die Entwicklung der Dauerlarve verläuft unterschiedlich zur normalen Larve, da die Organe sich an die neuen Umweltbedingungen anpassen müssen.

C. elegans ist ein ausgezeichnetes Modell, um die inter- und intrazellulären Signalwege zu analysieren, welche die Entwicklung steuern. Wir studieren vor allem die Entwicklung der Vulva des Hermaphroditen, welche für die Eiablage notwendig ist. Unter normalen Bedingungen ist die Entwicklung der Vulva invariabel und wird durch das Zusammenspiel dreier Signalwege reguliert; des RAS/MAPK, des NOTCH und des WNT Signalweges. Diese drei Signalwege interagieren miteinander und werden genauestens reguliert, um das invariable Zellteilungsmuster sicherzustellen, das zur Bildung einer funktionierenden Vulva notwendig ist. In dieser Arbeit habe ich untersucht, wie die Umweltbedingungen, insbesondere die Qualität der vorhandenen Nahrung, zusammen mit dem Insulin Signalweg die Entwicklung der Vulva beeinflussen. Das Züchten von *C. elegans* auf verschiedenen Nährmedien mit unterschiedlichen

Bakterienstämmen hat gezeigt, dass ein Bakterienstamm mit hohem Nährwert (z. B. Comamonas DA 1877) die Aktivität des RAS/MAPK Signalweges verstärkt im Vergleich zu Würmern, die auf Nährmedium mit dem üblichen *E. Coli* Stamm OP50 gewachsen sind. Dieser Effekt ist unabhängig vom Insulin Signalweg. Im ersten Teil meiner Arbeit habe ich hingegen gefunden, dass der Insulin Signalweg den RAS/MAPK Signalweg während der Vulvaentwicklung aktiviert. Durch eine genaue Epistase Analyse habe ich gezeigt, dass drei Hauptkomponenten des Insulin Signalweges, der Insulinrezeptor DAF-2, die Phosphatidyl-Inositol-3-Kinase (PI3K) AGE-1 und das homologe des menschliche PTEN Tumorsuppressors, DAF-18, die Entwicklung der Vulva unabhängig von weiter unten (downstream) liegenden Komponenten des kanonischen Insulin Signalweges regulieren. Zudem zeigte sich, dass der Einfluss des Insulinrezeptors DAF-2 auf den RAS/MAPK Signalweg teilweise unabhängig von PI3K AGE-1 ist und dass der hemmende Effekt von PTEN DAF-18 auf das RAS/MAPK Signal teilweise unabhängig von DAF-2 und AGE-1 ist. Zusammenfassend kann gesagt werden, dass die *C. elegans* Vulvaentwicklung ein einfaches aber relevantes Modell für die Untersuchung der wichtigsten Signalwege ist, welche eine entscheidende Rolle bei der Entwicklung und der Krebsentstehung spielen. Diese Arbeit zeigt insbesondere, welchen Effekt die Ernährung auf Signalwege haben kann, die zur Krebsentstehung beitragen. Zusätzlich konnte ich die Wirkung von neuen Moderatoren der RAS/MAPK Signalkaskade wie z. B. dem Insulinrezeptor DAF-2 aufklären.

In einem dritten Projekt, welches hauptsächlich von der Masterstudentin Debora Kehrli unter meiner Leitung ausgeführt wurde, haben wir das FLP Rekombinase System verwendet, um sehr spezifische Expression des Poly A bindenden Proteins PAB-1 in den primären Zellen der sich entwickelnden Vulva zu erzeugen. Dieses neue Werkzeug erlaubt uns, Gene zu finden, die in der Entscheidung des primären Zellschicksals in der Vulva involviert sind. Dieses dritte Projekt befindet sich nun in der Endphase.

Summary

Multicellular organisms are in constant interaction with the environment, sensing the conditions around them and reacting accordingly. The response to changes in the environmental conditions can be observed at several levels. At the level of the organism itself, for example when humans sweat in the heat and move to the shadow, at the level of the organs, when the heart rate increases during exercise, or at the level of the cells, when apoptosis is induced by external radiation or by the different checkpoints during cell division.

How the model organism *C. elegans* responds to environmental cues can also be studied at the different levels. The worm constantly searches for the best growth conditions. If the food is nutritional enough the worm stops foraging and eating, but if the food is not rich enough it constantly searches for food and its pharynx constantly pumps, taking in as much food as possible. If there is no food at all, the worm enters a long-lived alternative third larval stage, called the dauer stage. The individual organs develop differently when the worm enters the dauer stage, thus adjusting to the altered environmental conditions. Finally, at the cellular level, *C. elegans* serves as an excellent model to study inter- and intracellular signaling pathways during development. For this purpose we are investigating the development of the egg-laying organ of the hermaphrodite, the vulva. Under normal growth conditions, the development of the vulva is invariant and is regulated by three major signaling pathways, the RAS/MAPK, the NOTCH and the WNT pathways. The activities of these pathways are tightly regulated and crosstalk between them allows a precise and invariant pattern of divisions to occur, which is necessary to form a functional vulva. In this work, I used different mutations that affect vulval development to examine how environmental conditions, in particular the quality of the food provided to the worm together with the insulin pathway affect vulval development. By growing the worms on different strains of bacteria, I found that more nutritious bacteria (e.g. comamonas DA1877) enhance RAS/MAPK signaling compared to the standard *E. coli* strain OP50. This effect was independent of the insulin pathway. In the first part of my work, however, I found that the insulin pathway activates RAS/MAPK signaling during vulval development. By using detailed epistasis analysis, I found

that the three major components of the insulin pathway, the insulin receptor DAF-2, the Phosphatidyl-Inositol-3-Kinase (PI3K) AGE-1 and the homolog of the mammalian tumor suppressor PTEN DAF-18 each affect vulval development independently of further downstream components of the canonical insulin pathway. Furthermore, the DAF-2 effect was partly independent of AGE-1, and the DAF-18 effect was partly independent of DAF-2 and AGE-1. By using *C. elegans* vulval development as a simple yet representative model to study the major developmental pathways that play crucial rules during human development and cancer, this work shows how food can affect cancer related signaling pathways. I have also been able to identify significant new modulators of RAS/MAPK dependent vulval development, such as the insulin receptor.

In another project, performed mainly by Debora Kehrli under my supervision, we used the FLPase system to drive highly specific expression of the Poly A Binding protein PAB-1 in the primary cells of the developing vulva. This new tool allows us to identify the genes expressed in the primary cell lineage. This project is now in its final steps.

Abbreviations

1°	Primary
2°	Secondary
AC	Anchor Cell
C. elegans	*Caenorhabditis elegans*
CR	Caloric Restriction
DAF-c	DAuer Formation constitutive
DAF-d	DAuer Formation defective
DpMPK-1	Diphosphorylated MPK-1
DTC	Distal Tip Cells
E. coli	*Escherichia Coli*
EGF	Epidermal Growth Factor
EMS	Ethane Methyl Sulfonate
FAK	Focal Adhesion Kinase
FLP	Flippase
FRT	FLP Recognition Targets
GEF	Guanine Exchange Factor
gf	*gain of function*
GFP	Green Fluorescent Protein
InsR	Insulin Receptor
Let	Lethal
lf	*loss of function*
Lin	Lineage defective
MAPK	Mitogen-activated protein kinase
Muv	Multivulva
NICD	Notch IntraCellular Domain
PAB	Poly A Binding
PHTS	PTEN hamartoma tumor syndromes
$PI(3,4,5)P_3$ / PIP_3	PhosphatidylInositol(3,4,5)-trisphosphate
$PI(4,5)P_2$ / PIP_2	PhosphatidylInositol(4,5)-trisphosphate

PI3K	phosphatyidlinositol-3-kinase
PTEN	Phosphatase and TENsin homologue
RAS	RAt Sarcoma
rf	*reduction of function*
RNAi	RNA interference
RTK	Receptor Tyrosine Kinase
SHC	Src Homology domain Containing
UNC	Uncoordinated
VNC	Ventral Neuron Cord
VPC	Vulva Precursor Cell
Vul	Vulvaless

1) General Introduction to C. elegans [1]

1.1) Establishment of C. elegans as model organism

The nematode *Caenorhabditis elegans* (*C. elegans*), which was originally established as a model organism by Sydney Brenner in 1974 [2] is a simple, small, free living worm inhabiting microbe-rich habitats, in particular decaying plant matter. Several traits make *C. elegans* a very successful model organism. First of all, despite the obvious differences between humans and worms, about 36% of the *C. elegans* proteins have human orthologs [3], with human genes often being able to rescue mutants in the homologous worm genes [4]. Furthermore, the small size and lack of pigmentation make live microscopy of the whole worm very easy without any further preparation. Sulston et al. [5] were able, already in 1977, to determine the complete lineage of all the somatic cells of the adult hermaphrodite worm. The lineage of the somatic cells is invariant, and analysis of deviation from this lineage can help to better understand the function of different genes and their mutations. Furthermore, worms can be very easily and cheaply maintained in the laboratory, and can even be frozen and thawed dozens of years later. The worm exists mostly as an XX hermaphrodite, with the occasional appearance of an X0 male due to spontaneous non-disjunction of the X chromosome during meiosis in the germ line. Thanks to the relatively short life cycle and large progeny, more rapid research can be done (see "Life cycle" for details, chapter 1.2). The adult hermaphrodite can lay around 300 eggs during its lifetime, and each descendant is a genetic clone of the mother while males allow genetic crosses to combine different genetic backgrounds and thus create diversity.

Over the years, many genetic and molecular tools were developed to investigate how the different genes act alone and in coordination with other genes to allow for precise and invariant pattern of division and cell fate decisions. Either forward (mutagenesis) [2] or reverse genetic screens, in particular RNAi screens can be relatively easily performed with *C. elegans* [6], and thanks to the short life span and large progeny, a large number of mutants can be screened in a short time. Furthermore, since the worm reproduces by self-fertilization, clones of

each mutant are easily obtained. The genome of *C. elegans*, which contains approximately 100 million base-pairs which encode for about 20,000 genes. The *C. elegans* genome was the first genome of a multi-cellular organism to be completely sequenced. Several techniques allow for rapid and precise identification of the mutated genes [7-9]. Once the mutated gene is identified, rescue experiments can be performed with remarkable ease by injection of the wild-type gene into the gonad of the hermaphrodite. Even a simple PCR product of the gene can be used without any further cloning or processing, and the progeny of the injected hermaphrodite will express the introduced gene [10]. Furthermore, fusion PCR products of translational and transcriptional reporters can also be injected without any further cloning or processing. Live microscopy to follow the reporters can then be performed while the embryo or worm develop under the microscope. Injection of constructs in this manner leads to generation of extra-chromosomal multi-copy arrays. Such arrays are inherited in a mosaic manner, such that part of the progeny carries the array and part does not. Also, within the individual worm, some of the cells express the array and some do not. This allows performing mosaic analysis of the expression pattern, and finding out the cell in which the gene is required to give a proper rescue [11]. Integration of DNA fragments in the worm's genome can also be performed, either as multi-copy arrays [12]or as single copies [13].

1.2) Life cycle

The *C. elegans* life cycle is fixed and short. The normal growth temperature is between 15°C and 25°C, and the rate of development depends on the temperature in which the worms are grown. The higher the temperature, the faster the worm develops. At 20°C, embryogenesis occurs within about 18 hours. The worm then develops to adulthood by passing through four larval stages, termed L1-L4, with molting steps at the end of each developmental larval stage (Figure 1). These developmental stages (from hatching till first egg laid) take another approximately 2.5 days under normal conditions. However, if the plate is crowded or out of food, the worm goes through an alternative long-lived

developmental stage called the dauer larva stage (see "Dauer Development and The Insulin Pathway" below for details, chapter 2.1.1). Although the worm hatches with many of its organs and tissues developed and functional, during the different larval stages the development of other tissues still proceeds. For example, at the L1 stage the gonad primordium is composed of only four cells, termed Z1-Z4. At later developmental stages these cells divide, where Z1 and Z4 give rise to the somatic gonad and Z2 and Z3 give rise to the germline [14]. Another tissue that develops during the different larval stages is the vulva.

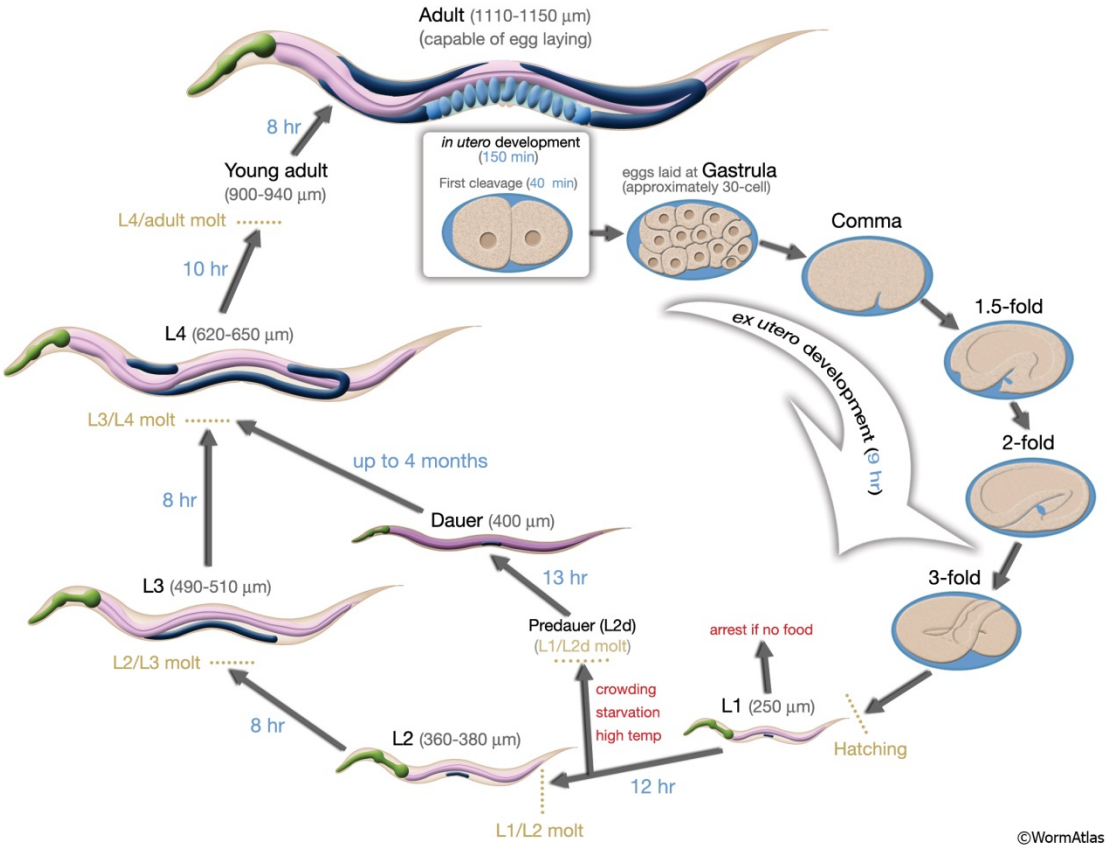

Figure 1. Life cycle of *C. elegans*. After fertilization of the egg, in-utero development proceeds till around 30 cells, at which time the egg is laid and the egg continues its development ex-utero. As the egg hatches at the L1 stage, a crucial decision occurs, and the worm developmental path is chosen based on environmental conditions. If the conditions are favorable, development continues through the L2-L4 stages till adulthood. However, under conditions of low food, crowding and/or high temperature, the worm develops into the dauer state, in which it will stay up to four months or until the conditions improve. Adapted from WormAtlas

1.3) Vulval development [15]

Like most organisms, the worm exists basically to eat and reproduce. To fulfill such basic needs, the anatomic structure of the hermaphrodite is very simple, composed of digestive, nervous and reproductive systems (Figure 2). The reproductive system is made of the somatic gonad, the germline and the egg laying and mating apparatus, the vulva.

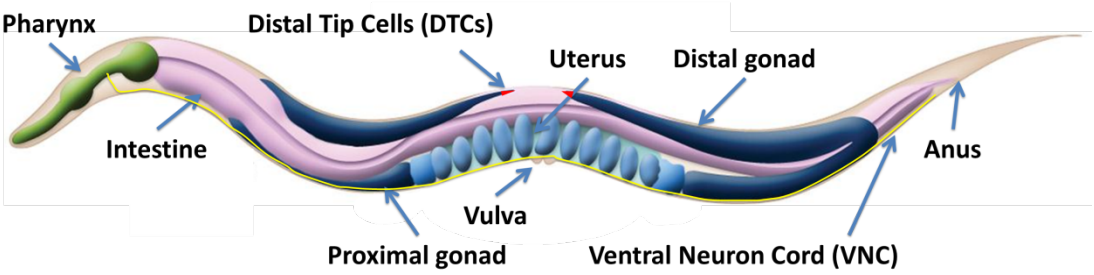

Figure 2. Schematic drawing of the hermaphrodite *C. elegans* anatomy. The anatomy of the worm is made of the pharynx, which is connected to the gut that goes throughout the worm's body, until it opens at the anus. The adult hermaphrodite has also two symmetrical U shaped gonadal arms extending laterally from the midline, where the uterus and vulva are. Finally, about a third of the somatic cells are neuronal cells, many of them in the head area, with a ventral neuron cord running along the length of the worm. Adapted from WormAtlas.

The invariant development of the vulva provides researchers with an excellent tool to examine how inter- and intra-cellular signals regulate organogenesis [16]. Vulva development occurs in a stepwise manner.

During the L1 and L2 stages, six out of the twelve Pn.p cells in the ventral hypodermis of the worm are specified as Vulva Precursor Cells (VPCs). These six cells (termed P3.p till P8.p) all have the potential to be induced to create a vulva. However, upon vulval induction during the second larval stage, only three cells (P5.p, P6.p and P7.p) are induced to form the vulva, while the remaining cells (P3.p, P4.p and P8.p) remain un-induced, divide once and fuse with the surrounding hyp7 hypodermis tissue (Figure 3). Deviation from this normal pattern of induction due to different mutations can lead to hyper-induction, where more than three cells are induced, and the generation of extra pseudo-

vulvae, a phenotype called Multi-vulva (Muv) or to hypo-induction, where fewer than three cells are induced, a phenotype called Vulvaless (Vul).

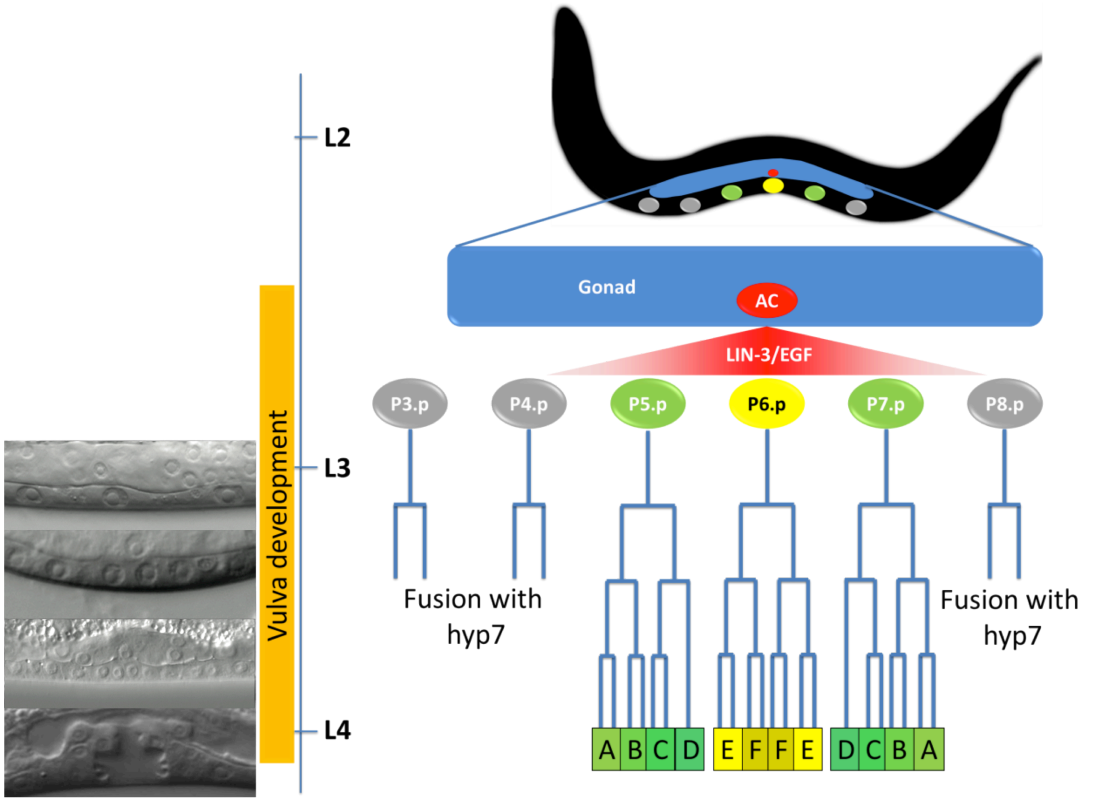

Figure 3. Vulval induction and development. During the second larval stage the AC starts releasing the EGF signal LIN-3 leading to vulval induction and division of the VPCs. P6.p receives the highest concentration of LIN-3 and adopts the 1° cell fate, giving rise to 8 cells with sub-fates termed VulE and VulF. P5.p and P7.p adopt the 2° cell fate, each one of them gives rise to 7 cells with sub-fates termed VulA, VulB1, VulB2, VulC and VulD. The remaining cells, P3.p, P4.p and P8.p remain un-induced, divide once and fuse to the surrounding hypodermis. (Pictures of VPCs from Ivo Rimann)

Production of the inductive signal begins at the middle of the second larval stage, at which time a specialized cell in the gonad called the Anchor Cell (AC) starts releasing the Epidermal Growth Factor (EGF) LIN-3 in a gradient fashion [17]. All six VPCs express the EGF receptor LET-23, however, P6.p, which is located directly below the AC, receives the highest concentration of LIN-3 and the RAS LET-60/MAPK MPK-1 signaling cascade is activated, which among other

processes described below, leads to increased levels of LET-23 [18]. The LET-60/MPK-1 pathway is composed, as mentioned, of the ligand LIN-3 that binds and thus leads to dimerization and activation of the Receptor Tyrosine Kinase (RTK) LET-23. In order to be exposed to the LIN-3 signal, LET-23 needs to be localized to the basolateral side of the cell membrane. A scaffolding complex composed of CASK/LIN-2, VELIS/LIN-7 and MINT/LIN-10 maintains this localization [19]. LET-23 auto- and trans-phosphorylates tyrosine residues on its intracellular domain, which serves as docking sites to adaptor proteins such as GRB2/SEM-5 via its SH2 domain. SEM-5 has also an SH3 domain, which binds proline rich domains, such as the carboxy-terminal domain of the RAS-Guanine Exchange Factor (GEF) SOS-1. Recruitment of SOS-1 to the membrane via SEM-5 allows it to load the GTPase LET-60 with GTP, thus activating it. Activated LET-60 then leads to recruitment and activation of the MAPKKK RAF/LIN-45 – MAPKK MEK/MEK-2 – MAPK ERK/MPK-1 cascade. An activated MPK-1 can regulate targets in the cytosol, as well as enter the nucleus to phosphorylate different transcription factors [20]. The ELK1/LIN-1 HNF3/LIN-31 transcription factors complex negatively regulates vulval induction. Upon activation of MPK-1, LIN-1 is phosphorylated which makes it inactive, and the vulval induction signal is transduced in P6.p [21].

LET-60 signaling in P6.p causes the cell to adopt the primary (1°) cell fate as well as to induce the secondary (2°) cell fate in the neighboring P5.p and P7.p by lateral signaling. LET-60 signaling leads to the production of the Notch receptor ligands LAG-2, APX-1 and DSL-1 in P6.p [22]. The NOTCH/LIN-12 receptor in the neighboring P5.p and P7.p binds these ligands, and is then cleaved to generate the notch Intra-Cellular Domain (NICD). The NICD then enters the nucleus, and together with the transcription factor CSL/LAG-1 regulates different genes involved in inhibition of LET-60 signaling in these cells such as the MPK-1 phosphatase LIP-1 [23] and indirectly also the activity of the EGFR phosphatase DEP-1 [24]. Notch signaling in 2° cells is also sufficient for induction of 2° fate [25], however, as to date, no genes are known that directly induce 2° cell fate downstream of the Notch pathway (see “the FLPase system”, chapter 4).

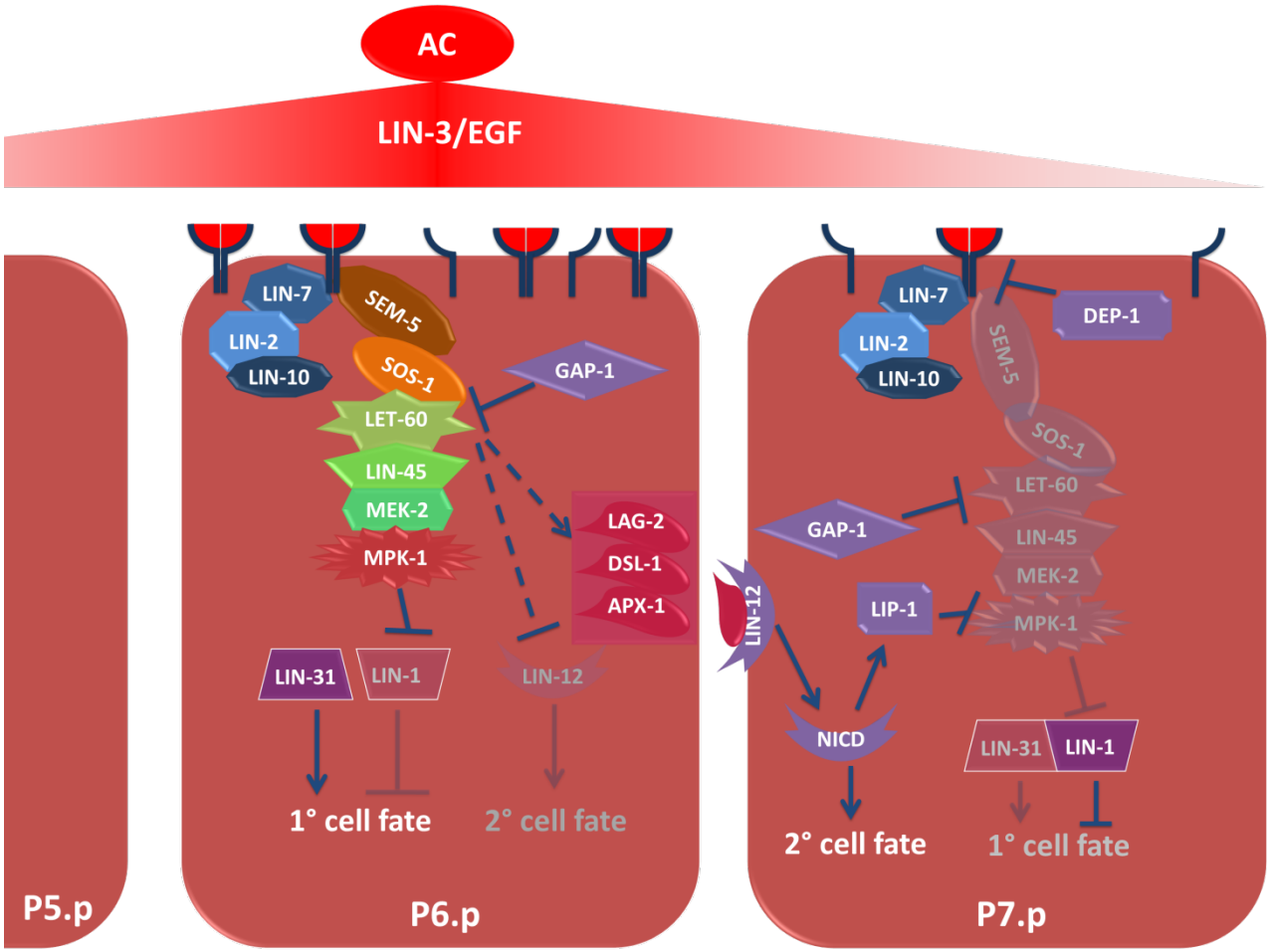

Figure 4. Overview of the inductive and lateral signals of vulval development. As the AC releases the LIN-3 ligand, LET-23 receptor molecules located on the baso-lateral side of the VPCs receive the signal. P6.p, located directly below the AC is exposed to the highest concentration of LIN-3, and activates the LET-60 RAS signaling cascade, which leads to the cell adopting the primary cell fate. In parallel, a lateral notch signal is generated by the production and release of the notch receptor ligands. The neighboring P5.p and P7.p receive the lateral signal which induces them to adopt the 2° cell fate while inhibiting MPK-1 signaling.

Also important in the process is the activity of LIN-3, LET-23 and LET-60 in the secondary cells. All VPCs are exposed to the LIN-3 signal and they all express LET-23, although at lower levels [26] and with faster internalization [18] of LET-23 compared to P6.p. Evidence exist to suggest that internalized EGFR continues to signal and that the signal generated by EGFR varies depending on its sub-cellular localization [27]. In *C. elegans,* it has been proposed that besides the lateral signal, also the LIN-3 gradient effects cell fate decisions [26].

Furthermore, a recent report showed that LET-60 can act in 2° cells via RalGDS to induce the 2° cell fate together with notch [28]. It is thus possible that rapid LET-23 internalization in P5.p and P7.p promotes alternative usage of LET-60 effectors to help drive the cells to adopt the 2° cell fate.

At the start of vulval induction none of the VPCs have divided. After induction, the induced VPCs go through three rounds of cell division with invariant timing and pattern of division to give rise to 22 cells (8 cells from the 1° cell and 7 cells from each of the 2° cells), which form the final mature vulva during the phase of vulval morphogenesis.

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2) PTEN negatively regulates MAPK signaling during C. elegans vulval development

2.1) Introduction

2.1.1) Dauer Development and The Insulin Pathway [1]

Under standard conditions (i.e. ample food, space and optimal temperature), *C. elegans* develops from egg to adult within 3 days, and has a life span of 2-3 weeks. However, under conditions of starvation, over-crowding or high temperatures, several signal transduction pathways act independently and in cooperation with each other to change the normal life cycle of the worm, and rather than proceeding from the L2 larval stage to the L3 larval stage, the L1 larva enters an alternative L3 stage called the dauer larva stage. The dauer larva is an arrested, long lived, non-feeding, stress resistant stage that can survive harsh conditions for several months until the environmental conditions improve, at which point the animal exits from dauer and development continues at the L4 stage. The dauer larva is distinct from the normal L3 larval stage in several aspects. It is much thinner, movement is limited and the pharynx does not pump. Further examination reveals an internal plug covering the oral opening and a specialized cuticle, which combined with the lack of pumping, confers resistance to harsh environmental conditions, including 1% SDS treatment. SDS resistance is often used for dauer isolation in genetic dauer formation analysis [2]. Another major difference between dauer and L3 larvae is the metabolic pathways used. As the worm passes from L1 to L2 and later developmental stages, the energy consumption as well as food intake increase and the animal changes its metabolic mechanism from the anabolic glyoxylate cycle based to aerobic respiration [3]. However, dauer larvae maintain the usage of the anabolic metabolism similar to the L1 stage, which can better fit the non-feeding, non-developing state of the worm.

Several signaling pathways regulate entry into and exit from dauer, namely the TGFβ-like, Insulin-like, guanylyl cyclase, Notch and hormonal signaling pathways. Mutations in these pathways can lead to either a Dauer Formation

constitutive (DAF-c) phenotype, where the worms enter dauer even under standard conditions, or a Dauer Formation defective (DAF-d) phenotype, where the worms cannot enter dauer even if the plate is starved and crowded.

The environmental cues that regulate dauer development are sensed by these pathways which transduce the decision making signal. For example, the worm senses how crowded the plate is by sensing the concentration of the dauer pheromone that is constitutively produced by its neighboring worms. High population density leads to high concentration of the pheromone that induces dauer formation and prevents recovery from dauer. Food, on the other hand, creates an opposite signal that inhibits dauer formation and the relative amounts of pheromone vs. food rather than the absolute amounts affect dauer decision.

The decision to enter dauer is more of a process rather than a switch. It begins at the L1 stage, which is the only stage that is responsive to the pheromone signal. At this stage, if the worm turns towards dauer formation, a slightly different L2 stage develops, a stage referred to as L2d, where the change to aerobic metabolism does not occur. Interestingly, until the L2d molt, the dauer decision can be reversed if the worms are no longer exposed to the pheromone but rather to food. However, worms that have entered the normal L2 stage are committed to normal development and cannot enter dauer [4]. Dauer larvae are referred to as a non-aging stage, and the life span of the worm after recovering from dauer is independent of how long the worm spent in dauer.

Of prime importance among the aforementioned pathways that regulate dauer formation is the insulin-like pathway, which affects not only dauer development but also the longevity of the worm. Animals with reduced insulin signaling can live as much as twice as long as wild-type worms [5]. Interestingly, reduction in insulin signaling extends life span not only in *C. elegans*, but the effect is conserved in flies and mice [6].

The insulin pathway, like most signaling pathways, begins with a ligand, in this case the insulin or insulin-like ligand. Remarkably, there are about 40 different insulin/insulin-like genes in *C. elegans* with only one described receptor, the RTK DAF-2. Surprisingly, not all ligands inhibit dauer formation and over-expression of some of them in fact induces dauer [7]. The mammalian Insulin Receptor (InsR) shares some functional similarities with other RTKs such as the EGFR (see

"Crosstalks between the RAS/MAPK and insulin pathways", chapter 2.1.3) yet its main function, as its name suggests, is to activate the insulin pathway. In *C. elegans*, active DAF-2 transduces the insulin signal by activating the PhosphatidylInositol 3 Kinase (PI3K) AGE-1. AGE-1 then phosphorylates phosphatidylinositol(4,5)-bisphosphate [$PI(4,5)P_2$] to PhosphatidylInositol-(3,4,5)-trisphosphate [$PI(3,4,5)P_3$], which acts as a secondary messenger. The main targets of PIP_3 are the serine/threonine kinases AKT-1, AKT-2 and SGK-1 that transduce the signal to further downstream targets, specifically phosphorylating and thus inhibiting the FOXO transcription factor DAF-16 [8, 9]. In the absence of food, the insulin pathway is inactive and the unphosphorylated-active DAF-16 enters the nucleus to regulate genes involved in dauer formation causing the worm to enter the dauer stage [10]. DAF-18, the ortholog of the human tumor suppressor PTEN, antagonizes the insulin pathway by dephosphorylating PIP_3 back to PIP_2 [11]. Loss of DAF-18 leads to hyper-activation of the insulin pathway, thus allowing AKT-1/2 to constitutively phosphorylate DAF-16 and as a result the worm cannot enter the dauer stage (DAF-d). In contrast, loss of function of *age-1* subsequently leads to inactivity of the insulin pathway and causes a DAF-c phenotype.

However, things are not as straightforward as they may appear. For example, although *akt-1(gf)* or *daf-18(rf)* can fully rescue the DAF-c phenotype of *age-1(lf)*, they do not fully rescue *daf-2* strong reduction of function *e1370* [12]. Furthermore, *akt-1/akt-2/sgk-1* mutants do not induce dauer in the same manner as *daf-2*, when combined with *daf-16(lf)* animals expressing a *daf-16* construct mutated in its phosphorylation sites [9]. Thus, strong evidence indicates that DAF-2 acts not only via the canonical insulin pathway to regulate dauer development.

As mentioned above, the insulin pathway affects not only dauer decision at early developmental stages, but also the life span of the adult worm. In fact, the regulations of these two processes are temporally independent of each other. For example, treatment with *daf-2* RNAi only during development does not extend life span, whereas starting *daf-2* RNAi treatment at the beginning of adulthood does extend life span to a similar extent as starting the RNAi treatment on the eggs and continuing throughout adulthood. Similar results were obtained with

daf-16 RNAi. Initiating *daf-16* RNAi on *daf-2(e1370)* animals during young adulthood completely reversed the effect of the *daf-2* mutation [10]. Thus, DAF-2 and DAF-16 affects life span only during the adult stage and not during development.

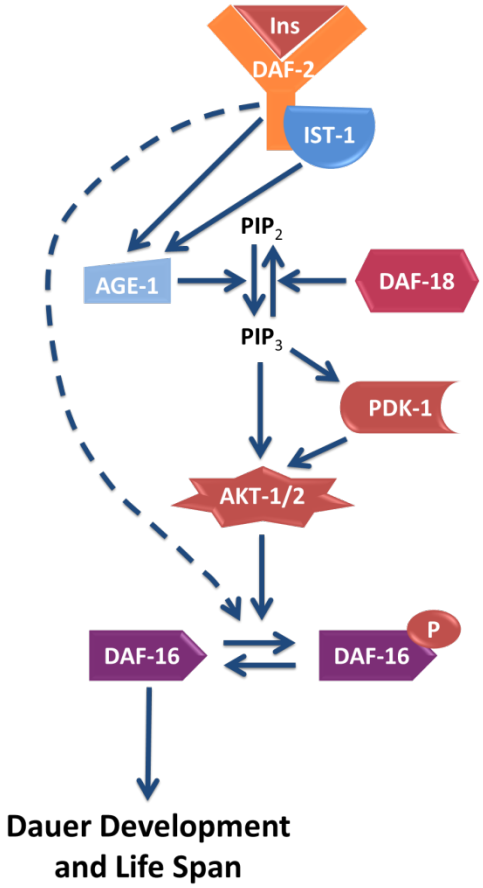

Figure 5. The canonical insulin pathway in *C. elegans*. The conserved insulin pathway in the worm consists of approximately 40 different insulin ligands, which bind the DAF-2 insulin receptor. Upon activation, DAF-2 activates the PI3-kinase AGE-1 either directly or via IST-1. AGE-1 then phosphorylates PIP2 to generate the secondary messenger PIP3, which leads to the activation of the serine/threonine kinases PDK-1 and AKT-1/2. Activated AKT proteins can then phosphorylate, and thus inhibit the FOXO transcription factor DAF-16, which is responsible for regulating genes involved in dauer formation. To antagonize the whole pathway, DAF-18 acts as a phosphatase that dephosphorylates PIP_3 back to PIP_2. Finally, DAF-2 has been shown to regulate via an unknown pathway DAF-16 in an AGE-1/AKT independent manner.

2.1.2) DAF-18/PTEN

Of special interest to this study is the ortholog of the human tumor suppressor PTEN, DAF-18. PTEN (Phosphatase and TENsin homologue) is the second most frequently mutated tumor suppressor gene in human cancer, often occurring in glioblastoma, melanoma, prostate and endometrial neoplasia [13]. Germline mutations in PTEN are also known to cause a variety of rare syndromes, collectively known as the PTEN hamartoma tumor syndromes (PHTS) [14]. Cowden syndrome is the best-described syndrome within PHTS, with approximately 80% of patients carrying germline PTEN mutations [15]. The main reported function of PTEN is as a lipid phosphatase, dephosphorylating PIP_3 at position 3, thus antagonizing the Insulin pathway [16]. However, PTEN can also act as a tyrosine and serine/threonine dual specificity protein phosphatase. The phosphatase core domain of PTEN (amino acids 123-131) is composed of several key amino acids, mutations of which affect the efficiency and specificity of the phosphatase domain [17]. One such mutation is G129E, which causes PTEN to lose its lipid phosphatase activity while retaining most of its protein phosphatase activity [18] and [17]. Using the G129E mutation, numerous recent reports have provided strong evidence for the crucial role of the PTEN protein phosphatase activity in regulating cell migration, invasion and spreading, in a manner that is independent of the activation of the canonical insulin pathway. For example, PTEN G129E was reported to affect migration, spreading and focal adhesions of cultured cells as well as *in-vitro* binding and dephosphorylation of the Focal Adhesion Kinase FAK [19]. Furthermore, PTEN protein phosphatase inhibits glioblastoma cell invasion accompanied by a decrease in phosphorylation of FAK, while having no effect on the phosphorylation of the PH-domain-containing serine/threonine kinase Akt [20]. PTEN protein phosphatase activity also inhibits tumor invasion in nude mice [21], to modulate Shc dependent cell motility and to directly bind and dephosphorylate the adapter protein Shc *in-vitro* [22].

DAF-18, the *C. elegans* ortholog of PTEN, is involved in regulation of longevity, entry into the dauer stage and cell-cycle arrest [12, 23]. Human PTEN can functionally replace DAF-18 in the worm and rescue its DAF-d phenotype [24].

However, several reports suggest that DAF-18 in the worm does more than simply regulating the canonical insulin pathway. In a whole genome RNAi screen for synthetic lethality of DAF-18, Suzuki and Han found several genes that cause synthetic lethality or sterility in a manner that is independent of DAF-16 [25]. Among these genes was *ceh-18,* which is involved in the sperm-sensing mechanism dependent on the gonadal sheath cells that inhibits oocyte maturation via MPK-1 phosphorylation. *vab-1* was shown previously to synergize with *ceh-18* [26], and the researchers examined how VAB-1 might interact with DAF-18. *vab-1* RNAi in *daf-18(lf)* animals caused an increase in DpMPK-1 levels compared with *daf-18(lf)* or *vab-1* RNAi and the researchers concluded that DAF-18 and VAB-1 act in the same pathway but also in parallel of each other to inhibit MPK-1 activation. Furthermore, DAF-18 was recently shown to act as a protein phosphatase on top of its well-described lipid phosphatase activity. A recent publication found that DAF-18 directly binds and dephosphorylates the ephrin receptor VAB-1, affecting oocyte maturation in an AGE-1 independent manner [27]. Unlike the aforementioned research, the authors further propose that VAB-1 negatively regulates DAF-18, and that DAF-18 positively regulates phosphorylation of MPK-1, thus *vab-1(lf)* animals displayed increased levels of phosphorylated MPK-1 (DpMPK-1) while *daf-18(lf)* and *vab-1(lf); daf-18(lf)* animals displayed reduced DpMPK-1.

Another interesting aspect of DAF-18 in *C. elegans* is its involvement in stress resistance. Treatment of *C. elegans* with a sub-lethal dose of a stressor (a process called hormesis) leads to extended life span as well as enhanced stress resistance. *daf-2* and *age-1* mutants have higher thermo-tolerance and life span after hormesis than wild-type, while mutations in *daf-18, daf-16* or *daf-12* do not have extended life span. However, *daf-16* and *daf-12* do not show a defect in thermo-tolerance after hormesis, while *daf-18* is required for full induction of thermo-tolerance after hormesis [28]. Thus, DAF-18 appears to act in a DAF-16 independent manner in inducing thermo-tolerance after hormesis. In another publication, *smg-1* was found to regulate life span in a *daf-18* dependent manner via *daf-16* and in parallel to *daf-2* and *age-1* [29], again, providing evidence for DAF-18 to act independently of the canonical insulin pathway.

2.1.3) Crosstalks between the RAS/MAPK and insulin pathways

Although the RAS/MAPK and insulin pathways are considered distinct pathways that regulate different processes in *C. elegans*, a rather strong interaction between the two pathways was described in mammals, with many of the key components regulating and interacting with each other. For example, one of the key downstream effectors of RAS is PI3K, which is known to directly bind and be activated by RAS [30]. A similar situation is quite possible in *C. elegans*, since AGE-1 contains a LET-60 binding domain [31]. Furthermore, the InsR, similar to the EGFR, acts as a docking domain for components of the RAS/MAPK pathway either via GRB2 or, more commonly in the case of the InsR, via the adaptor protein SHC, which then recruits GRB2 to activate the pathway [32, 33]. Finally, AKT has been found to phosphorylate and thus inhibit RAF [34, 35]

In *C. elegans*, very little work has been done to investigate the relationship between these two pathways. Work from our laboratory showed *daf-2(e1370)* to suppress the Muv phenotype of *let-60(gf)* [36], an observation that helped start the current work. More detailed analysis was performed by Nanji et. al where *let-60(gf)* was found to rescue the DAF-c phenotype of *daf-2(rf)* and *age-1(lf)* mutants [37].

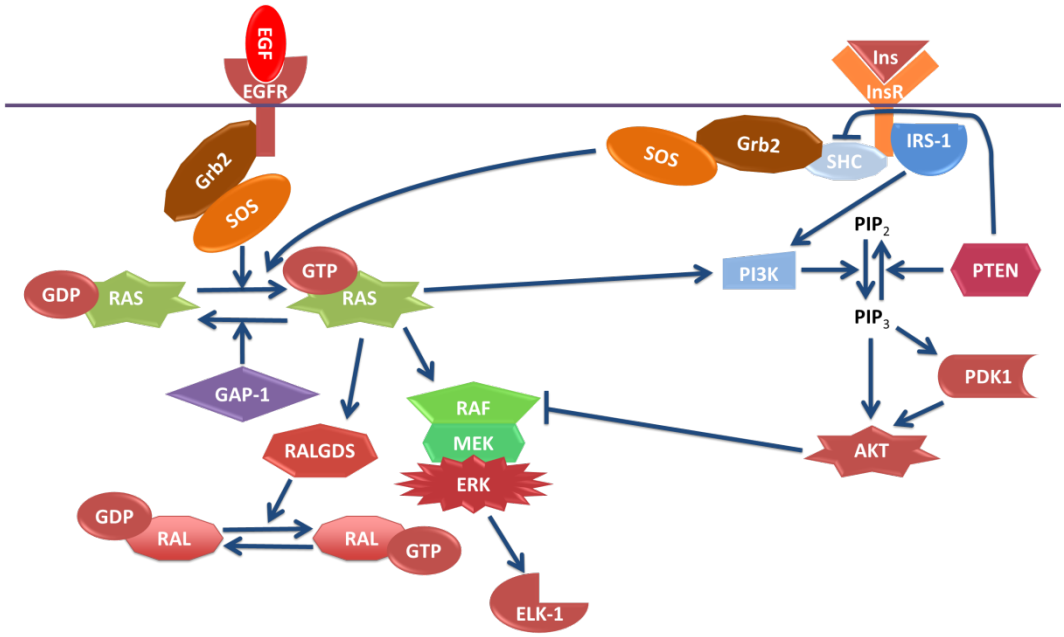

Figure 6. Mammalian based model for the crosstalk between the RAS/MAPK pathway and the insulin pathway. The two pathways regulate each other on key levels, where RAS can directly bind and activate PI3K and the insulin receptor can lead to activation of RAS by recruiting SHC and GRB2. Downstream components of the pathway regulate each other as well, such as the inhibition of AKT on RAF as well as different interactions of further downstream elements not shown in this figure.

2.2) Aim of this project

The RAS/MAPK, Notch and WNT pathways act together to regulate vulval development. Research in recent years has advanced our understanding of how these three signaling pathways regulate the different cell fates not only within the individual cells but also via crosstalk between the different pathways across the cells. Previous work from our laboratory has shown that DAF-2 and environmental conditions such as starvation, could also affect vulval development. In this work I first examined which components of the insulin pathway are involved in regulating vulval development. I then focused my analysis on understanding the negative regulation of RAS/MAPK signaling by the PTEN homolog DAF-18.

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2.3) Manuscript draft: PTEN negatively regulates MAPK signaling during C. elegans vulval development

PTEN negatively regulates MAPK signaling during C. elegans vulval development

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Key words: Signaling; PTEN; MAP kinase; C. elegans.

Running title: C. elegans PTEN inhibits MAPK

Abstract

Vulval development in C. elegans serves as an excellent model to examine the crosstalk between different conserved signaling pathways that are deregulated in human cancer. The concerted action of the EGFR/RAS/MAPK, WNT and Notch pathways determines an invariant pattern of cell fates in three vulval precursor cells. We have discovered and characterized a novel form of crosstalk between components of the insulin and EGFR/RAS/MAPK pathway. The insulin receptor DAF-2 stimulates while DAF-18 PTEN inhibits RAS/MAPK signaling in vulval precursor cells. Surprisingly, part of the inhibitory activity of DAF-18 PTEN on the RAS/MAPK pathway is independent of its PIP_3 lipid phosphatase activity and does not involve further downstream components of the insulin pathway. Additional results indicate that DAF-18 negatively regulates vulval induction through its protein phosphatase activity downstream of RAS, most likely by inhibiting MAPK activation in the nucleus. Thus, single mutations in the PTEN tumor suppressor may result in simultaneous hyper-activation of several oncogenic signaling pathways.

Introduction

PTEN (Phosphatase and TENsin homologue) is the second most frequently somatically mutated- or lost- tumor suppressor gene in human cancer, often occurring in glioblastoma, melanoma, prostate and endometrial neoplasia [1]. Germline mutations in PTEN are also known to cause a variety of rare syndromes, collectively known as the PTEN hamartoma tumor syndromes (PHTS) [2]. Cowden syndrome is the best-described syndrome within PHTS, with approximately 80% of patients carrying germline PTEN mutations [3]. The main reported function of PTEN is as a lipid phosphatase, which dephosphorylates PhosphatidylInositol(3,4,5)-trisphosphate (PIP_3) at position 3, thereby antagonizing the insulin pathway [4]. However, PTEN can also act as a dual-specificity tyrosine and serine/threonine protein phosphatase. The catalytic phosphatase domain of PTEN (amino acids 123-131) contains several conserved amino acids, mutations of which affect the efficiency and specificity of the phosphatase activity [5]. One such mutation is G129E, which causes PTEN to lose its lipid phosphatase activity while retaining most of its protein phosphatase activity [4, 6]. Using the G129E mutation, numerous reports have provided strong evidence for a crucial role of PTEN protein phosphatase activity in regulating cell migration, invasion and spreading independently of the canonical insulin signaling pathway. For example, PTEN G129E regulates cell migration, spreading and the formation of focal adhesions [7]. Moreover, PTEN G129E binds and de-phosphorylates the Focal Adhesion Kinase FAK in vitro [7]. In glioblastoma cells injected into nude mice, PTEN G129E inhibits cell invasion, accompanied by decreased FAK phosphorylation, while the activation of the Akt kinase is not changed by PTEN G129E expression [8, 9]. PTEN also binds and dephosphorylates the adaptor protein Shc to modulate Shc-dependent cell motility [10].

Under favorable growth conditions, C. elegans larvae pass through four distinct larval stages termed L1 to L4. However, under conditions of starvation or overcrowding, the L1 larvae enter a long-lived, stress resistant alternative developmental stage called the dauer larva. The C. elegans *daf-18* gene encodes the single PTEN ortholog that controls entry into the larval dauer stage, life span

and cell-cycle progression, mainly by inhibiting the insulin signaling pathway [11, 12]. Human PTEN can functionally replace C. elegans DAF-18 to rescue the *daf-18(lf)* DAuer Formation defective (DAF-d) phenotype [13].

In the presence of abundant food, binding of various insulin ligands to the DAF-2 insulin receptor causes the activation of the AGE-1 phosphatyidlinositol-3-kinase (PI3K)[14]. AGE-1 phosphorylates $PI(4,5)P_2$ to $PI(3,4,5)P_3$, which acts as a second messenger. Two main targets of PIP_3 are AKT-1 and AKT-2 that transduce the insulin signal further downstream by phosphorylating and thereby inhibiting the FOXO transcription factor DAF-16 [15]. In the absence of the insulin signal, non-phosphorylated DAF-16 enters the nucleus to activate genes promoting entry into the dauer stage [16]. The main reported function of DAF-18 PTEN is to antagonize the insulin pathway by dephosphorylating PIP_3 [12]. Loss of *daf-18* thus leads to hyper-activation of the insulin pathway and a DAF-d phenotype, while loss of *daf-2* or *age-1* function leads to a DAuer Formation constitutive (DAF-c) phenotype.

Recent evidence indicates that similar to mammalian PTEN, C. elegans, DAF-18 can also act as a protein phosphatase to regulate insulin-independent events. For example, DAF-18 PTEN directly binds and dephosphorylates the ephrin receptor tyrosine kinase VAB-1 to regulate oocyte maturation in the gonads [17]. Moreover, multiple genes causing synthetic lethality in combination with *daf-18(lf)* have been identified, pointing a additional functions of DAF-18 besides its role in insulin signaling [18].

The development of the C. elegans hermaphrodite vulva, the egg-laying organ, is one of the best-characterized models for organogenesis [19]. The interplay between the conserved RAS/MAPK, NOTCH and WNT signaling pathways specifies two vulval cell fates. Beginning in the L2 stage, the gonadal Anchor Cell (AC) releases the EGF ligand LIN-3, which activates in the six vulval precursor cells P3.p through P8.p (VPCs) that are aligned along the ventral midline the EGF receptor homolog LET-23. The VPC located nearest the AC (P6.p) receives most of the inductive LIN-3 EGF signal and hence exhibits the strongest activity of the RAS/MAPK pathway, allowing P6.p to adopt the primary (1°) vulval cell fate. Consequently, P6.p produces a lateral signal that activates the LIN-12 NOTCH signal in the neighboring VPCs P5.p and P7.p. Notch signaling in these two VPCs

induces them to adopt the secondary (2°) cell fate and at the same time blocks transduction of the inductive LIN-3 signal by inhibiting MAPK activation. The 1° VPC P6.p and the 2° VPCs P5.p and P7.p each go through three rounds of cell division to generate 22 cells that form the vulva. The remaining three distal VPCs (P3.p, P4.p and P8.p) adopt the non-vulval 3° fate, which is to divide once and then fuse with the surrounding hypodermis (hyp7).

Mutations in genes encoding components of the signaling pathways, which regulate vulval induction, change the patterning of the VPC fates, which can readily be quantified. For example, mutations that hyperactivate the RAS/MAPK pathway cause the induction of more than three and up to six VPCs, resulting in a phenotype called Multivulva (Muv).

In this work, we have discovered and characterized a new mode of crosstalk between components of the insulin and the RAS/MAPK pathway. Using genetic epistasis analysis, we found that the insulin receptor DAF-2 stimulates while DAF-18 PTEN inhibits RAS/MAPK signaling in the VPCs. Surprisingly, part of the inhibitory activity of DAF-18 on the RAS/MAPK pathway is independent of its PIP_3 lipid phosphatase activity and does not involve further downstream components of the insulin pathway. Our results indicate that DAF-18 negatively regulates vulval induction most likely by directly inhibiting MAP kinase MPK-1 signaling.

Results

daf-18 inhibits vulval induction independently of the canonical insulin signaling pathway

In our previous work, we had found a crosstalk between the DAF-2 insulin receptor and the RAS/MAPK signaling pathways during vulval development [20]. To further investigate the interaction between these two signaling pathways, we performed a systematic epistasis analysis by combining various mutations in the two signaling pathways and quantifying the levels of vulval induction (Table 1). As reported previously, a rf mutation in the insulin receptor daf-2 partially suppressed the Muv phenotype of *let-60 RAS(gf)* animals (Table 1, rows 1,2) [20]. Conversely, a *loss-of-function (lf)* mutation in the PTEN homolog *daf-18* strongly enhanced the *let-60(gf)* Muv phenotype (Table 1, row 3). Moreover, *daf-18(lf)* suppressed the Vul phenotype caused by mutations in the EGF receptor *let-23* or in its cofactor *lin-2*, which activates the LET-60 RAS/MAPK signaling pathway in the VPCs (Table 1, rows 4-7). Since DAF-18 PTEN counteracts the type I phosphatidyl-inositol-3 kinase (PI3K) AGE-1 that transduces the insulin signal downstream of DAF-2, we tested if an *age-1(lf)* mutation could revert the enhanced vulval induction caused by *daf-18(lf)*. Surprisingly, *age-1(lf)* only partially suppressed the increase in vulval induction caused by *daf-18(lf),* both in the *let-60(gf)* and the *lin-2(lf)* backgrounds (Table 1, rows 8,9). Also, the *daf-2(rf)* mutation only partially reverted the enhancement of the *let-60(gf)* Muv phenotype by *daf-18(lf)* (Table 1, row 10), suggesting that DAF-18 inhibits vulval induction to some extent independently of DAF-2 and AGE-1. Moreover, mutations in further downstream components of the DAF-2 insulin pathway had no detectable effect on vulval induction. For example, a *gf* mutation in *akt-1*, which encodes one of the two AKT homologues transducing the insulin signal downstream of AGE-1, did not suppress the *let-23(rf)* Vul phenotype, and a *lf* mutation in *daf-16*, which encodes a FOXO transcription factor that is negatively regulated by the insulin pathway, did not enhance the *let-60(gf)* Muv phenotype (Table 1, rows 11, 12).

We further tested the role of AGE-1 during vulval development. Since *age-1(lf)* leads to a constitutive dauer phenotype (DAF-c) that is maternally rescued,

homozygous *age-1(lf)* worms could only be analyzed in the F1 progeny of heterozygous *age-1(lf)/+* parents or when rescued by the *daf-18(lf)* or *daf-16(lf)* mutations. Our analysis indicated that *age-1* also exhibits a partial maternal rescue in vulval induction, since the homozygous *age-1(lf); let-60(gf)* progeny obtained from heterozygous *age-1(lf)/+* parents displayed similar levels of vulval induction as *let-60(gf)* single mutants, while homozygous *age-1(lf); let-60(gf)* double mutants maintained in the *daf-16(lf)* background exhibited a partially suppressed Muv phenotype, though to a lesser extent than *daf-2(rf); let-60(gf)* double mutants (Table 1, rows 13, 14, p-value ≤0.05).

Taken together, the genetic analysis indicates that the DAF-2 insulin receptor promotes and DAF-18 PTEN inhibits vulval induction. DAF-2 and DAF-18 both regulate vulval induction through AGE-1-dependent as well as AGE-1-independent pathways that do not utilize the canonical insulin pathway downstream of AGE-1.

DAF-18 inhibits vulval induction independently of other PI3Ks

AGE-1 is the only C. elegans member of the type I family of PI3Ks, which convert $PI(4,5)P_2$ into $PI(3,4,5)P_3$. To further investigate the AGE-1-independent effect of DAF-18 on vulval induction, we tested whether alternative PI3Ks might generate PIP_3 in the absence of AGE-1. *vps-34* encodes a type III PI3K, which catalyzes the production of $PI(3)P_1$, and *piki-1* encodes a type II PI3K involved in the engulfment of apoptotic cell corpses [21]. In order to examine the role of these alternative PI3Ks during vulval induction, we performed RNAi against *vps-34* and *piki-1* in *age-1(lf); daf-18(lf); lin-2(lf)* animals and tested for a further reduction of vulval induction. RNAi to *vps-34* and *piki-1* has been previously shown to be effective [22, 23]. Neither *vps-34* nor *piki-1* RNAi caused any significant reduction in the number of induced VPCs when compared to control (gfp) RNAi animals (Table 2). It thus appears unlikely that an alternative PI3K acts redundantly with AGE-1 during vulval induction, suggesting that DAF-18 regulates vulval induction not only by regulating PIP_3 levels but also via a lipid phosphatase-independent activity.

daf-18 inhibits 1° vulval cell fate specification

To characterize the nature of the cell fate transformation caused by *daf-18(lf)*, we quantified the levels of the EGL-17::CFP reporter, whose expression is induced by RAS/MAPK signaling in the 1° vulval cell lineage. In the wild-type, the two VPCs P5.p and P7.p that exhibit highest levels of LIN-12 Notch activity and hence adopt the 2° cell fate only very weakly expressed EGL-17::CFP while in *daf-18(lf)* single mutants the levels of EGL-17::CFP were slightly elevated in P5.p and P7.p (Figure 1G). Moreover, *daf-18(lf) let-60(gf)* double mutants displayed higher levels of ectopic EGL-17::CFP expression in the distal VPCs compared to *let-60(gf)* single mutants (Figure 1A-D and G) as well as an increased frequency of high EGL-17::CFP expression in adjacent proximal VPCs (P5.p through P7.p) (26% adjacent EGL-17::CFP in *daf-18(lf); let-60(gf)*, versus 12% in *daf-18(+); let-60(gf)*, n=23 and n=25, respectively). Besides the increase in EGL-17::CFP expression, the morphology of the vulval invagination at the L4 larval stage was changed in *daf-18(lf) let-60(gf)* double mutants. The morphology of the vulval invagination formed at the L4 stage by the P5.p to P7.p descendants of most *let-60(gf)* single mutants resembles the single invagination formed in the wild-type (Figure 1C). In *daf-18(lf) let-60(gf)* double mutants, on the other hand, the P5.p to P7.p descendants were often completely detached from the cuticle, resulting in an abnormal shape of the vulval invagination (Figure 1E, F) (37% detached P5.p and/or P7.p descendants in *daf-18(lf) let-60(gf)* versus 3% detached in *let-60(gf)*, n=54 and n=35, respectively). A detachment of the P5.p and P7.p descendants from the cuticle is indicative of a 2° to 1° cell fate transformation as it has been observed in mutants exhibiting elevated MAPK activity in the 2° lineage [24].

Thus, *daf-18(lf)* increases the number of VPCs adopting the 1° cell fate and causes a partial 2° to 1° fate transformation in P5.p and P7.p.

daf-18 negatively regulates vulval induction upstream or at the level of mpk-1

Since EGFR/RAS/MAPK signaling induces the 1° vulval cell fate and *daf-18(lf)* mutants exhibited an increased expression of the 1° cell fate marker EGL-17::CFP, we sought to determine at what level DAF-18 inhibits the activity of the EGFR/RAS/MAPK signaling pathway in the VPCs. For this purpose, we performed further epistasis analysis combining *daf-18(lf)* with mutations in different components of the RAS/MAPK pathway. *daf-18(lf)* increased the levels of vulval induction in most of mutants in the RAS/MAPK pathway tested, confirming that DAF-18 negatively regulates the RAS/MAPK signaling during vulva induction. For example, when combined with mutations in different positive regulators of the RAS/MAPK pathway such as *let-23(rf)*, *lin-2(lf)* and *lin-45(rf)*, *daf-18(lf)* significantly suppressed the Vul phenotype of these mutants (Table 3, rows 3-8). However, *daf-18(lf)* did not suppress the Vul phenotype of *lin-3(rf)* (table 3, rows 9, 10). In particular, *daf-18(lf)* suppressed a *lf* mutation in the RAS-GEF *sos-1* when assayed in the *let-60(gf)* background to rescue the lethality caused by *sos-1(lf)*, placing *daf-18* function downstream of *sos-1* (table 3, rows 11, 12). However, since vulval induction in *sos-1(lf); let-60(gf)* animals is still partly sensitive to the inductive anchor cell signal, we cannot exclude the possibility that DAF-18 might inhibit RAS/MAPK signaling through a SOS-1 independent branch of the pathway. Importantly, *daf-18(lf)* did not affect the completely Vul phenotype caused by *mpk-1(lf)* (table 3, rows 13, 14). *daf-18(lf)* also caused a very weak synthetic Muv when combined with *lf* mutations in negative regulators of the RAS/MAPK pathway such as *dep-1* and *gap-1*(Table 3, rows 15-18). However, since each of these single mutants display a 100% wild-type vulva phenotype, any deviation from the normal induction can be considered to be significant. Thus, DAF-18 inhibits RAS/MAPK signaling downstream of or in parallel with the RAS-GEF SOS-1 and upstream or at the level of the MAPK MPK-1.

daf-18 acts in the VPCs to inhibit vulval induction

To further investigate the role of DAF-18 during vulval induction, we constructed a translational reporter by inserting a *gfp* cassette in frame between the last exon and the 3' UTR into a genomic fragment encompassing 1.3 kb of 5' regulatory sequences and the complete *daf-18* coding sequences. This DAF-18::GFP reporter rescued both the dauer defective (DAF-d) phenotype (data not shown) as well as the vulval phenotypes of *daf-18(lf)* with similar efficiency as a 6.5kb genomic fragment spanning the entire *daf-18* locus (Fig. 3). DAF-18::GFP expression was observed in many tissues during all larval stages, specifically in the developing vulva as well as in the uterus, the ventral nerve cord and the distal tip cells (data not shown). In particular, equal levels of DAF-18::GFP expression were detected in the six VPCs of L2 larvae, and expression persisted in the descendants of the induced VPCs until the Pn.pxxx stage. Interestingly, the sub-cellular localization of DAF-18::GFP changed over the course of vulval development. In the VPCs of L2 larvae prior to and during induction (Pn.p stage), DAF-18::GFP was predominantly localized in the cytoplasm and the nucleus (Fig. 2 A, B and B'). However, at the subsequent stages (Pn.px to Pn.pxx stages), DAF-18::GFP became increasingly localized to the plasma membrane of the vulval cells (Figure 2 C, D and D'). Plasma membrane staining peaked at the "Christmas tree" (Pn.pxxx) stage, when almost all the protein appeared to be localized to the membranes and nuclear staining was reduced to barely detectable levels (Figure 2, E and F). Since the DAF-18::GFP reporter was widely expressed also in tissues surrounding the vulval cells, we examined whether DAF-18 acts cell-autonomously in the VPCs to regulate vulval induction. For this purpose, we expressed *daf-18 cDNA* fused with *gfp* under control of the Pn.p cell-specific *lin-31* promoter, which is active in the VPCs before and during vulval induction (*Plin-31::daf-18cDNA::gfp::unc-54 3' UTR*). Indeed, introduction of the *lin-31::daf-18::gfp* transgene into *daf-18(lf); let-23(rf)* animals repressed vulval induction with similar efficiency as the *daf-18::gfp* reporter or a *genomic daf-18* rescue construct (Fig. 3). Thus, DAF-18 acts cell-autonomously in the VPCs to inhibit RAS/MAPK signaling during vulva induction.

Both lipid and protein phosphatase activities of DAF-18 inhibit vulval induction.

Mammalian PTEN acts as a lipid phosphatase as well as a dual-specificity protein phosphatase [6, 9]. A recent report has shown that also C. elegans DAF-18 can act as a protein phosphatase inhibiting signaling by the VAB-1 ephrin receptor during oocyte maturation [17]. The G129E mutation in the catalytic center of human PTEN eliminates the lipid phosphatase activity, while retaining the protein phosphatase activity [5]. The corresponding glycine 174 residue in C. elegans DAF-18 was therefore mutated to glutamic acid in the *daf-18 genomic* rescue construct. To quantify the rescuing activity of the *daf-18* wild-type (*daf-18 wt*) and the G174E mutated lipid phosphatase mutant (*daf-18 G174E*), these constructs were expressed in the *daf-18(lf) let-60(gf)* and *let-23(rf); daf-18(lf)* backgrounds, and vulva induction was quantified. As expected, expression of *daf-18 wt* rescued both the DAF-d (data not shown) and vulval phenotypes of *daf-18(lf)* (Figure. 4). In contrast, *daf-18 G174E* did not rescue the DAF-d phenotype ([13] and own observation), yet exhibited a partial though significant rescuing activity on the vulval induction phenotype in three out of five independent lines that were assayed (Figure. 4). These results indicate that the DAF-18 protein and lipid phosphatase activities each play independent roles in negatively regulating the RAS/MAPK pathway and that both activities are required for full inhibition of vulval induction by DAF-18. *daf-18* mutants exhibit elevated levels of phosphorylated MAP kinase. Finally, we tested whether *daf-18(lf)* mutants exhibit elevated levels of activated, phosphorylated MAPK. For this purpose, Western blot of L4 whole worm extracts was probed for total and double phosphorylated MAPK (DpMPK-1) and the ratio between the DpMPK-1 and total MPK-1 signal intensities was calculated. While wild-type and *daf-18(lf)* L4 larvae contained only low levels of DpMPK-1 that could not be reliably quantified, *daf-18(lf) let-60(gf)* double mutants displayed significantly higher levels of DpMPK-1 as well as a two-fold increase in the ratio of DpMPK-1 to MPK-1 when compared to *let-60(gf)* single mutants (Figure 5). These results confirm that DAF-18 negatively regulates RAS/MAPK activity during larval development.

Discussion

Here, we have shown that different components of the Insulin pathway, which is a key regulator of development, reproduction and life span in metazoans, positively regulate RAS/MAPK signaling during *C. elegans* vulval development. In particular, signaling by the Insulin receptor DAF-2 positively regulates MAPK activation, though the effect of DAF-2 on vulval development does not involve activation of the canonical Insulin pathway. Surprisingly, DAF-2 signaling regulates vulval induction in two distinct ways, through an AGE-1 dependent and AGE-1 independent pathway. One possible explanation for the AGE-1-independent branch of DAF-2 signaling is supported by mammalian data, which suggest that the Insulin receptor can directly stimulate RAS activation by recruiting GRB2 and the SOS GEF [25, 26]. Moreover, we found that the *C. elegans* PTEN ortholog DAF-18 strongly inhibits RAS/MAPK signaling. Vulval induction in *daf-18(lf) let-60(gf)* double mutants reaches similar levels as seen in the strongest Muv mutants such as *lin-15AB(lf)* [27]. The increase in RAS/MAPK signaling in *daf-18(lf)* mutants could be partially reverted by loss of the PI3K activity, suggesting that elevated levels of PIP_3 do stimulated RAS/MAPK signaling but cannot explain all of the functions DAF-18 exerts during vulval induction. Accordingly, the inhibitory activity of DAF-18 requires both the lipid and protein phosphatase activities of DAF-18. PIP_3 acts as a second messenger that activates multiple downstream targets. One major PIP_3 target in the Insulin pathway is the AKT kinase, which phosphorylates and thereby inhibits the FOXO transcription factor DAF-16. However neither *akt-1* nor *daf-16* mutations had any detectable effect on vulval induction. Thus, PIP_3 must act via other targets to stimulate RAS/MAPK signaling. Increased levels of PIP_3 in the plasma membrane could, for example, enhance the recruitment of an alternative GEF that activates RAS signaling in parallel to the RAS-GEF SOS-1 [28]. Furthermore, we observed that prior to and at early stages of vulva induction, DAF-18::GFP was localized predominantly in the cytoplasm and nucleus of the VPCs, while membrane localization of DAF-18 only became apparent at later stages. Previous observation of mammalian PTEN localization suggested that PTEN performs different functions depending on its sub-cellular localization [29]. It has been

proposed that the lipid phosphatase activity is important for the cytoplasmic and membrane functions of PTEN, while the protein phosphatase activity is rather required for its nuclear functions [29, 30]. Nuclear localization of PTEN in mammalian cells is often associated with G1 arrest and accompanied by decreased levels of ERK phosphorylation. Indeed, Western blot analysis revealed elevated levels of DpMPK-1 in *daf-18(lf)* mutants, supporting our model that DAF-18 blocks MAPK activation during vulval induction. Prior to induction, the VPCs are maintained in G1 arrest and the timing of exit from the G1 arrest is important for proper vulval development [31]. It is possible that DAF-18 acts in the nucleus as a protein phosphatase to control gene transcription and cell cycle progression as part of the timing mechanism that regulates vulval development.

In humans, PTEN is one of the most frequently mutated tumor suppressor genes. However, not all disease phenotypes associated with loss of PTEN can be explained simply by hyper-activation of the Insulin pathway. Thus, PTEN must have other functions that are independent of its inhibitory activity in the Insulin pathway. Also, in *C. elegans*, Suzuki and Han [18] found that many synthetic phenotypes of *daf-18(lf)* mutants including embryonic lethality and sterility, which are independent of DAF-16 and do not involve DAF-2 signaling. Thus, our work provides further evidence to highlight the importance of DAF-18 PTEN in the range of processes it regulates and may serve as basis for further understanding of cancer progression and onset. Thus, single mutations in the PTEN tumor suppressor may result in the simultaneous hyper-activation of several oncogenic signaling pathways.

Materials and Methods

General worm methods

Standard methods were used for maintaining and manipulating Caenorhabditis elegans [32]. Animals were cultured at 20°C and the wild-type strain is the Bristol N2 strain. Information regarding the mutants used in this study can be found on WormBase (http://www.wormbase.org). Mutations used according to their linkage group:

LG I: *daf-16(mu86)*, LG II: *age-1(mg44), let-23(sy1), dep-1(zh34), unc-4(e120)* to cis link *dep-1(zh34)* LG III: *daf-2(e1370), mpk-1(ga117)*, LG IV: *let-60(n1046gf), daf-18(ok480), lin-3(n1417), lin-45(sy96)*, LG V: *akt-1(mg144gf)*, LG X: *lin-2(n397), sos-1(s1031), unc-46(e177)* to cis link *sos-1(s1031)*, LG X: *lin-2(n397), gap-1(ga133).*

Plasmids and PCR fusion constructs

pIN03 (*daf-18 genomic G174E*) was made by fusion PCR of two overlapping fragments of the whole genomic *daf-18* starting 1.3Kb upstream of the ATG and ending 0.5Kb downstream of the STOP using primers which contain the mutation G174E (GGC to GAA) in the overlapping region and cloning to pGEM-T

pIN05 (*daf-18 genomic wt*) was made by cloning the whole genomic fragment of *daf-18* starting 1.3Kb upstream of the ATG and ending 0.5Kb downstream of the STOP and cloning to pGEM-T

pIN17 (*Plin-31::daf-18 cDNA::gfp::unc-54 3'UTR*) was made by amplifying *daf-18 cDNA::gfp* from previously cloned plasmid with NotI sites on both ends, digestion with NotI and cloning to pB253.

daf-18 genomic translational reporter was made using fusion PCR of three parts by inserting a *gfp* cassette in frame between the last exon and the 3' UTR into a genomic fragment encompassing 1.3 kb of 5' regulatory sequences and the complete daf-18 coding sequences

Details on the primers used for the different constructs are (gladly) available upon request.

Transgenic lines

Worms expressing extra-chromosomal transgenic arrays were generated by microinjection of DNA into young adult worms [33]. pIN03 (zhEx344), pIN05 (zhEx382) and pIN17(zhEx358) were injected at a concentration of 50ng/ul. The fusion PCR daf-18 genomic::gfp (zhEx343) translational reporter was injected at a concentration of 30ng/ul. Co-marker used was either pCFJ90(Pmyo-2::mCherry) at a concentration of 2ng/ul or pTJ1157(Plin-48::gfp) at a concentration of 50ng/ul. Final concentration of injected DNA was filled up to 150ng/ul using the empty plasmid pBsKs.

Fluorescence microscopy

GFP and CFP expression were observed under fluorescent light illumination with either a Leica DMRA microscope equipped with a cooled CCD camera (Hamamatsu ORCA-ER) or Olympus BX61 with Q Imaging Fast 1394 Retiga 2000R camera (Q Imaging Inc., Canada) controlled by the Openlab 5 software (Improvision/PerkinElmer). Animals were mounted on 4% agarose pads in M9 solution with 20mM tetramisole hydrochloride. Quantification of fluorescence levels was performed under the same picture acquisition settings for all conditions examined.

Vulval induction

Vulval induction was scored by examining worms at the L4 stage under Nomarski optics as described [34]. The number of VPCs that had adopted a 1° or 2° Vulval fate was counted for each animal and the induction index was calculated by dividing the number of 1° or 2° induced cells by the number of animals scored. Statistical analysis was performed using a t-test for independent samples.

RNA interference analysis

RNA interference analysis (RNAi) was performed by feeding animals dsRNA-producing bacteria as described previously [35]. 10 P0 animals at the L1 larval stage were transferred to plates containing RNAi bacteria grown on 3mM IPTG.

Vulval induction was scored in the F1 progeny at the L4 larval stage to count the number of induced VPCs. GFP and *vps-34* RNAi clones were taken from the Ahringer library and *piki-1* RNAi clone was taken from the ORFeome library.

Western blot analysis

45 animals at the L4 stage were picked to 15ul of 1x SDS-SB, boiled at 95°C for 5min, centrifuged at 14,000rpm for 2 min and loaded on 10% acrylamide gels. Phosphorylated MPK-1 was blotted using the mono-clonal antibody M8159 (SIGMA) that specifically identifies the double phosphorylated ERK. Total MPK-1 was blotted using the ERK 2 (K-23) antibody (SANTA CRUZ BIOTECHNOLOGY). Quantification of the bands was performed using the ImageJ software [36].

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Figure Legends

Figure 1. DAF-18 inhibits 1° vulval fate specification.

Expression of the primary cell fate marker EGL-17::CFP analyzed by fluorescence (A and B) and Nomarski (C and D) microscopy of *let-60(gf)* (A and C) and *daf-18(lf) let-60(gf)* (B and D) animals at the 2 cell stage of vulval development. At the Christmas tree stage of vulva development P5.p and P7.p often maintain their wild-type appearance *in let-60(gf)* (E) while they usually detach from the cuticle in *daf-18(lf) let-60(gf)* animals (F). (G) represents quantification of EGL-17::CFP expression at the 2 and 4 cell stages of vulval development in different genetic backgrounds. %High/%Low/%Absent represents the fraction of the animals with High/Low/Absent levels of EGL-17::CFP expression. n≥20 in all experiments.

Figure 2. Expression pattern and sub-cellular localization of DAF-18::GFP

Nomarski (A, C and E) and fluorescence (B, D and F) images of animals expressing the DAF-18::GFP translational reporter at the 1 cell stage (A and B), 2-4 cell stage (C and D) and the Christmas tree stage (E and F).(B') and (D') represent magnification of the marked area.

Figure 3. DAF-18 expression in the Pn.p cells inhibits vulva induction

let-23(rf); daf-18(lf) animals expressing different extra-chromosomal constructs were allowed to grow till the L4 stage and vulval induction was quantified using Nomarski imaging. * indicates a p-value ≤0.05, ** indicates a p-value ≤0.005, *** indicates a p-value ≤0.0005. For each line the t-test was performed comparing the animals with and without the array from the same plate. n≥20 in all experiments.

Figure 4. A lipid phosphatase dead mutant of DAF-18 remains partially active

let-23(rf); daf-18(lf) animals expressing *daf-18 wt and daf-18 G174E* extra-chromosomal constructs were allowed to grow till the L4 stage and vulval induction was quantified using Nomarski imaging. * indicates a p-value ≤0.05, ** indicates a p-value ≤0.005, *** indicates a p-value ≤0.0005. For each line the t-test was performed comparing the animals with and without the array from the same plate

Figure 5. DAF-18 inhibits MPK-1 phosphorylation.

Whole worm extracts of wt*, daf-18(lf),* let-60(gf) and *daf-18(lf) let-60(gf)* animals at the L4 stage were blotted against total DpMPK-1 and MPK-1 (A). Intensity of each band in three independent experiments was quantified and the average ratio of DpMPK-1 to total MPK-1 was calculated (B)

Figure 6. Proposed model for crosstalk between RAS/MAPK and insulin pathways during vulval development.

The LET-60/MPK-1 pathway leads to MPK-1 entering the nucleus where it regulates transcription of genes controlling the 1° cell fate. The insulin pathway, mostly known to regulate dauer development and longevity, regulates the LET-60/MPK-1 pathway at several levels. DAF-2 enhances LET-60/MPK-1signaling by activating AGE-1, which leads to increase in PIP3 production, which probably enhances LET-60/MPK-1 signaling. Another effect of DAF-2 is recruitment of SEM-5 to the membrane, thus activating LET-60. DAF-18 can inhibit LET-60/MPK-1 signaling in two manners. Either by dephosphorylating PIP3 or via its protein phosphatase activity, which dephosphorylates as yet an unknown target.

Tables

Table 1. Epistasis analysis between the insulin and *RAS*/MAPK pathways.

	genotype	induction	% Vul	% Muv	n
1	*let-60(gf)*	4.16±0.89	0	79.1	283
2	*daf-2(rf); let-60(gf)*	3.24±0.63$^{***(1)}$	0	19.3	31
3	*daf-18(lf) let-60(gf)*	4.99±0.81$^{***(1)}$	0	96.4	221
4	*let-23(rf)*	0.55±0.92	93.8	0	129
5	*let-23(rf); daf-18(lf)*	1.90±1.21$^{***(4)}$	63.9	6.8	133
6	*lin-2(lf)*	1.34±1.22	73.8	0	42
7	*daf-18(lf); lin-2(lf)*	2.50±0.89$^{***(6)}$	35.5	1.7	59
8	*age-1(lf); daf-18(lf) let-60(gf)*	4.61±0.82$^{***(1)}$	0	94.7	113
9	*age-1(lf); daf-18(lf); lin-2(lf)*	2.15±1.11$^{***(6)}$	53.1	6.1	49
10	*daf-2(rf); daf-18(lf) let-60(gf)*	4.40±0.84$^{(1)}$	0	88.0	25
11	*let-23(rf); akt-1(gf)*	0.36±0.82$^{(4)}$	94.9	0	39
12	*daf-16(lf); let-60(gf)*	4.18±0.95$^{(1)}$	0	77.5	80
13	*age-1(lf); let-60(gf)* †	4.08±0.83$^{(1)}$	0	76.2	164
14	*age-1(lf); daf-16(lf); let-60(gf)*	3.62±0.71$^{**(1)}$	0	54.1	24

% Vul indicates the fraction of animals with less than three induced VPCs. % Muv indicates the fraction of animals with more than three induced VPCs. Induction indicates the average number of induced VPCs per animal. ** indicates a p-value ≤0.005; *** indicates a p-value ≤0.0005. Numbers in brackets indicates the row number against which a t-test was performed. Alleles used: LG I: *daf-16(mu86)*, LG II: *age-1(mg44), let-23(sy1)*, LG III: *daf-2(e1370)*, LG IV: *let-60(n1046), daf-18(ok480)*, LG V: *akt-1(mg144)*, LG X: *lin-2(n397)*. † F1 progeny of heterozygous *age-1(lf)/+* parents

Table 2. RNAi against alternative PI3Ks

	age-1(lf); daf-18(lf); lin-2(lf); RNAi	induction	% Vul	% Muv	n
1	*gfp*	1.59±1.41	71	12	17
2	*vps-34*	1.60±1.24	65	0	20
3	*piki-1*	1.69±1.25	69	6	16

% Vul indicates the fraction of animals with less than three induced VPCs. % Muv indicates the fraction of animals with more than three induced VPCs. Induction indicates the average number of induced VPCs per animal. Alleles used: LG II: *age-1(mg44)*, LG IV: *daf-18(ok480)*, LG X: *lin-2(n397)*.

Table 3. Epistasis analysis of daf-18 with components of the RAS/MAPK pathway.

	genotype	induction	% Vul	% Muv	n
1	wild-type	3.00±0.00	0.0	0.0	many
2	*daf-18(lf)*	2.99±0.10	0.9	0.0	107
3	*let-23(rf)*	0.55±0.92	93.8	0.0	129
4	*let-23(rf); daf-18(lf)*	1.90±1.21***(9)	63.9	6.8	133
5	*lin-2(lf)*	1.34±1.22	73.7	0.0	42
6	*daf-18(lf); lin-2(lf)*	2.50±0.89***(11)	35.5	1.7	59
7	*lin-45(rf)*	1.81±1.25	57.3	0.0	68
8	*daf-18(lf) lin-45(rf)*	2.29±1.11*(17)	39.5	2.3	42
9	*lin-3(lf)*	0.81±0.89	95.2	0.0	21
10	*daf-18(lf) lin-3(lf)*	0.46±0.74	94.1	0.0	34
11	*let-60(gf); sos-1(lf)*	2.60±0.91	16.7	0.0	30
12	*daf-18(lf) let-60(gf); sos-1(lf)*	4.19±0.68***(11)	0.0	90.3	31
13	*mpk-1(lf)*	0	100	0.0	14
14	*mpk-1(lf); daf-18(lf)*	0	100	0.0	17
15	*gap-1(lf)*	3.00±0.00	0.0	0.0	many
16	*daf-18(lf); gap-1(lf)*	3.05±0.25	1.0	7.9	101
17	*dep-1(lf)*†	3.00±0.00	0.0	0.0	18
18	*dep-1(lf)*†*; daf-18(lf)*	3.01±0.06	0.0	1.4	71

% Vul indicates the fraction of animals with three or less induced VPCs. % Muv indicates the fraction of animals with more than three induced VPCs. Induction indicates the average number of induced VPCs per animal. * indicates a p-value <0.05 *** indicates a p-value <0.0005, numbers in brackets indicates the row number against which a t-test was performed. Alleles used: LG II: *let-23(sy1)*, *dep-1(zh34)* LG III: *mpk-1(ga117)* LG IV: *daf-18(ok480), let-60(n1046), lin-3(n1417), lin-45(sy96)* LG V: *sos-1(s1031)* LG X: *lin-2(n397), gap-1(ga133)*. † *dep-1(zh34)* is cis-linked to *unc-4(e120)*, # *sos-1(s-1031)* is cis-linked to *unc-46(e177)*

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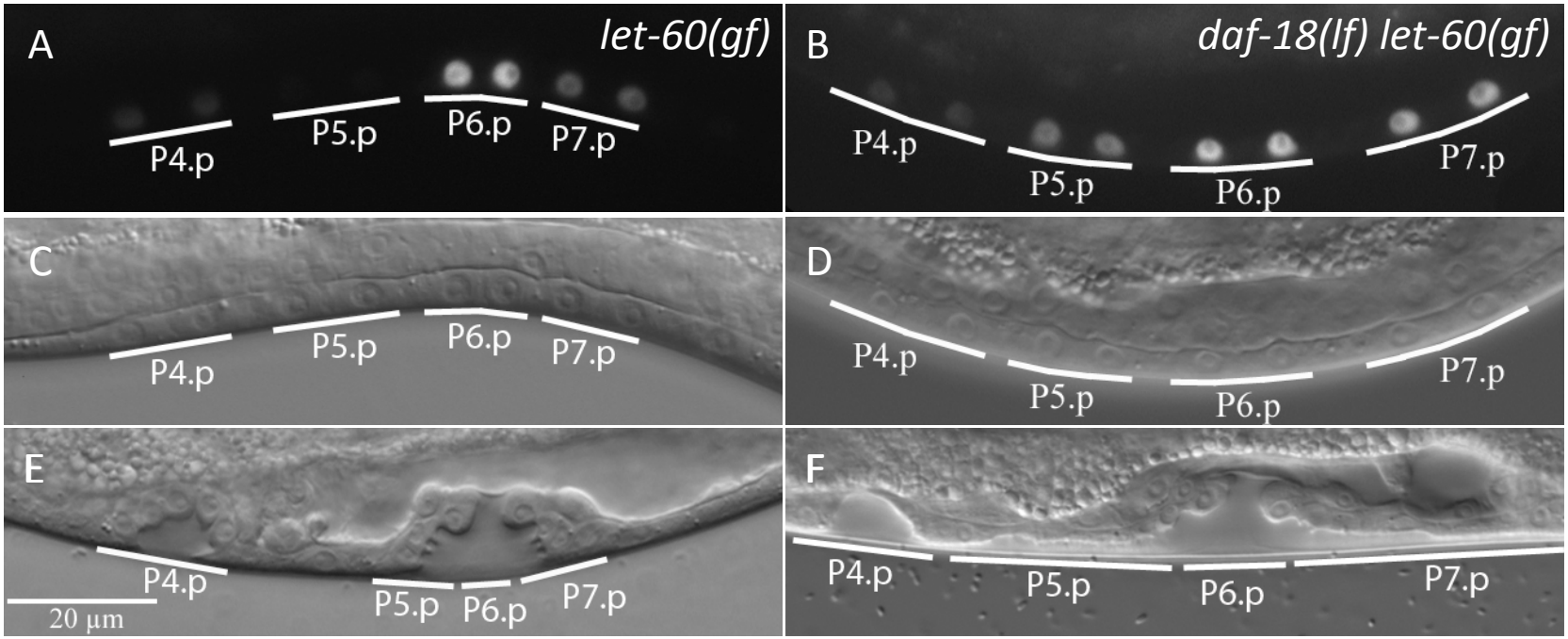


Figure 1. DAF-18 inhibits 1° vulval fate specification. Primary cell fate marker, egl-17::cfp, expression (A and B) and nomarski (C and D) images of *let-60(gf)* (A and C) and *daf-18(lf) let-60(gf) (B and D)* animals at the 2 cell stage of vulval development. At the christmas tree stage of vulva development P5.p and P7.p often maintain their wt appearance in *let-60(gf)* (E) while they usually detach from the cuticle in *daf-18(lf) let-60(gf)* animals (F). (G) represents quantification of egl-17::cfp expression at the 2 and 4 cell stages of vulval development in different genetic backgrounds. %High/%Low/%Absent represents the fraction of the animals with High/Low/Absent levels of EGL-17::CFP expression. n≥20 in all experiments.

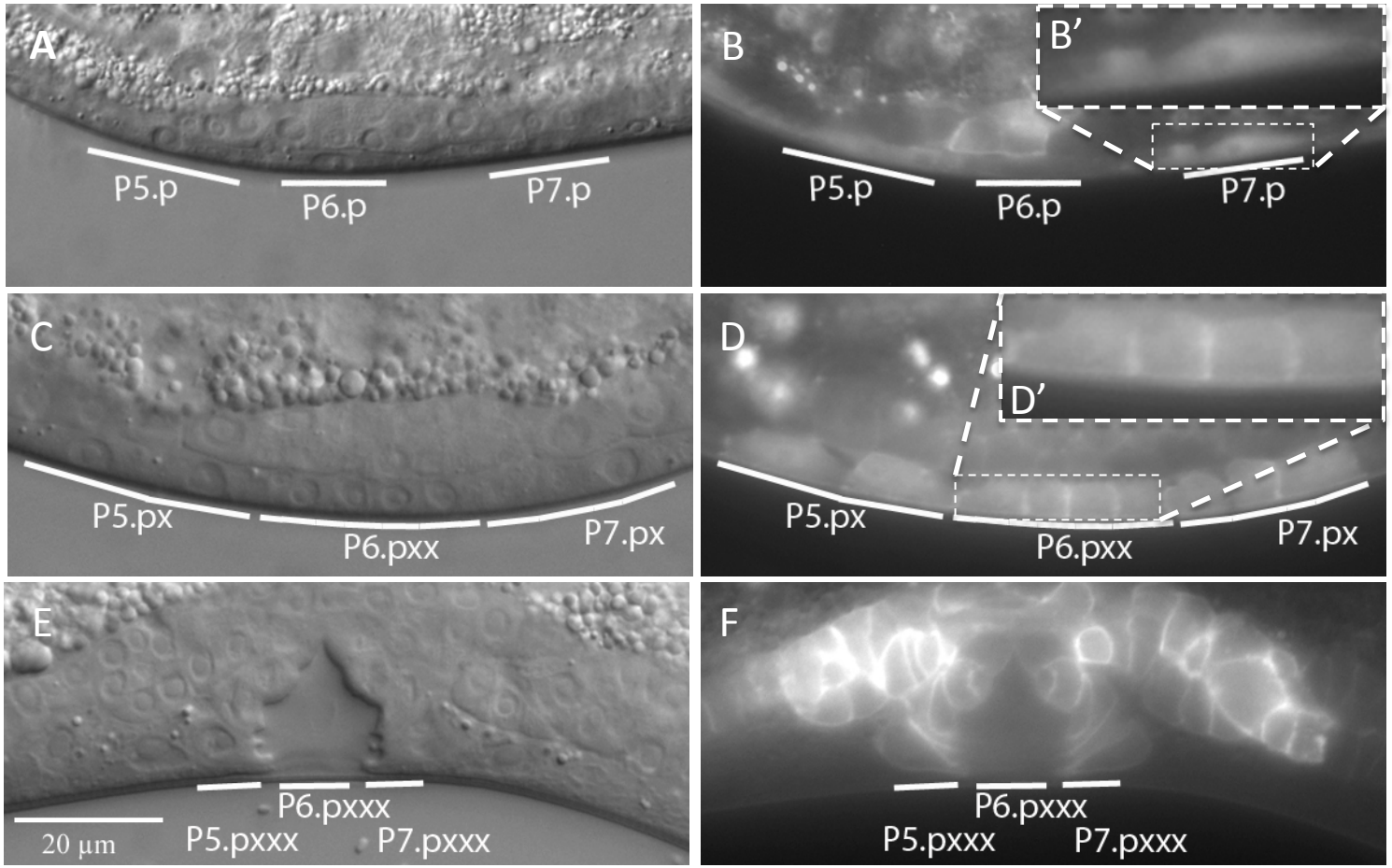
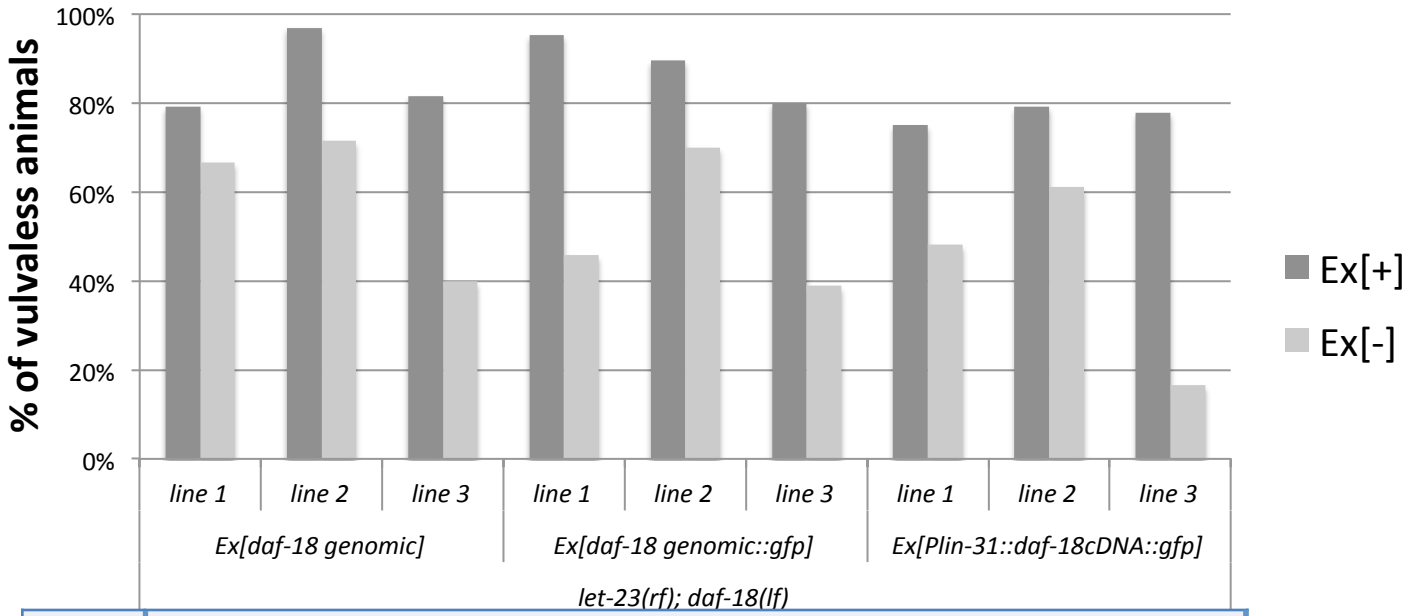
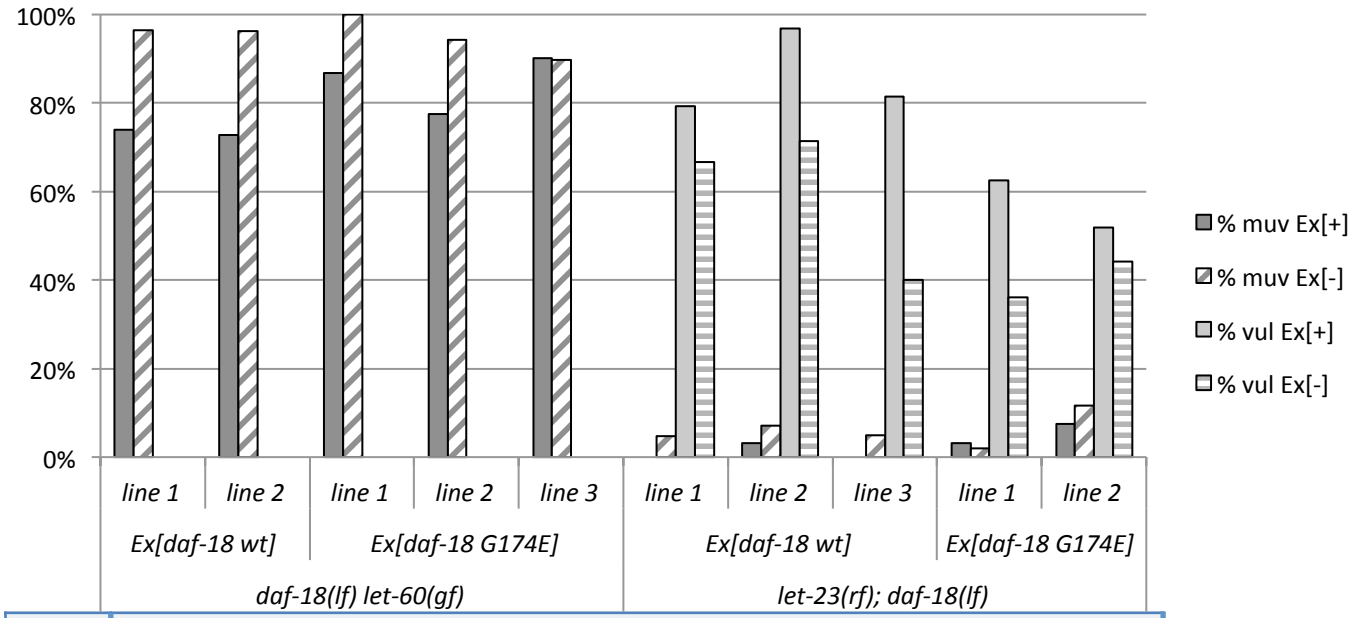


Figure 2. Expression pattern and subcellular localization of DAF-18::GFP. Nomarski (A, C and E) and fluorescence (B, D and F) images of animals expressing the DAF-18::GFP translational reporter at the 1 cell stage (A and B), 2-4 cell stage (C and D) and the christmas tree stage (E and F).(B') and (D') represent magnification of the marked area.



	Induction ±std								
Ex[+]	0.98 ±1.21	0.44 ±0.81	1.07 ±1.19	0.60 ±0.89	0.84 ±1.09	1.50 ±1.21	1.54 ±1.11	1.06 ±1.29	1.28 ±1.27
Ex[-]	2.19 ±0.8	1.88 ±1.15	2.38 ±1.02	2.42 ±0.94	1.90 ±0.91	2.47 ±0.92	2.19 ±1.06	2.11 ±1.18	2.89 ±0.27
t-test	***	***	***	***	*	*	*	**	***

Figure 3. daf-18 expression in the Pn.p cells inhibits vulva induction. *let-23(rf); daf-18(lf)* animals expressing different extra-chromosomal constructs were allowed to grow till the L4 stage and vulval induction was quantified using nomarski imaging. * indicates a p-value of ≤0.05, *indicates a p-value of ≤0.005, *** indicates a p-value of ≤0.0005. For each line the t-test was performed comparing the animals with and without the array from the same plate. n≥20 in all experiments.



	Induction ±std									
Ex[+]	3.85 ±0.68	3.82 ±0.72	4.52 ±0.96	4.42 ±0.98	4.65 ±0.84	0.98 ±1.21	0.44 ±0.81	1.07 ±1.19	1.84 ±1.20	2.10 ±1.20
Ex[-]	4.70 ±0.67	5.31 ±0.68	4.96 ±0.62	4.83 ±0.86	4.83 ±0.82	2.19 ±0.86	1.88 ±1.15	2.38 ±1.02	2.45 ±0.93	2.37 ±0.98
t-test	***	***	*			***	***	***	*	

Figure 4. A lipid phosphatase dead mutant of DAF-18 remains partially active. *let-23(rf); daf-18(lf)* animals expressing wt and G174E daf-18 extra-chromosomal constructs were allowed to grow till the L4 stage and vulval induction was quantified using nomarski imaging. * indicates a p-value of ≤0.05, ** indicates a p-value of ≤0.005, *** indicates a p-value of ≤0.0005. For each line the t-test was performed comparing the animals with and without the array from the same plate

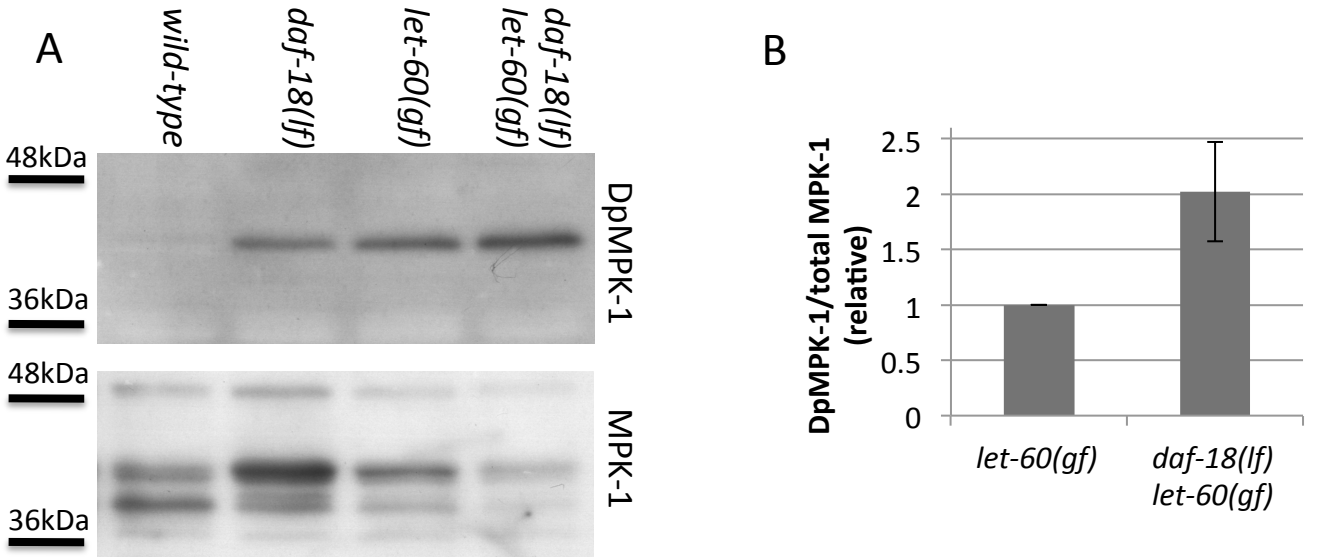


Figure 5. DAF-18 inhibits MPK-1 phosphorylation. Whole worm extracts of *let-60(gf)* and *daf-18(lf) let-60(gf)* animals at the L4 stage were blotted against total DpMPK-1 and MPK-1 (A). Intensity of each band in three independent experiments was quantified and the average ratio of DpMPK-1 to total MPK-1 was calculated (B)

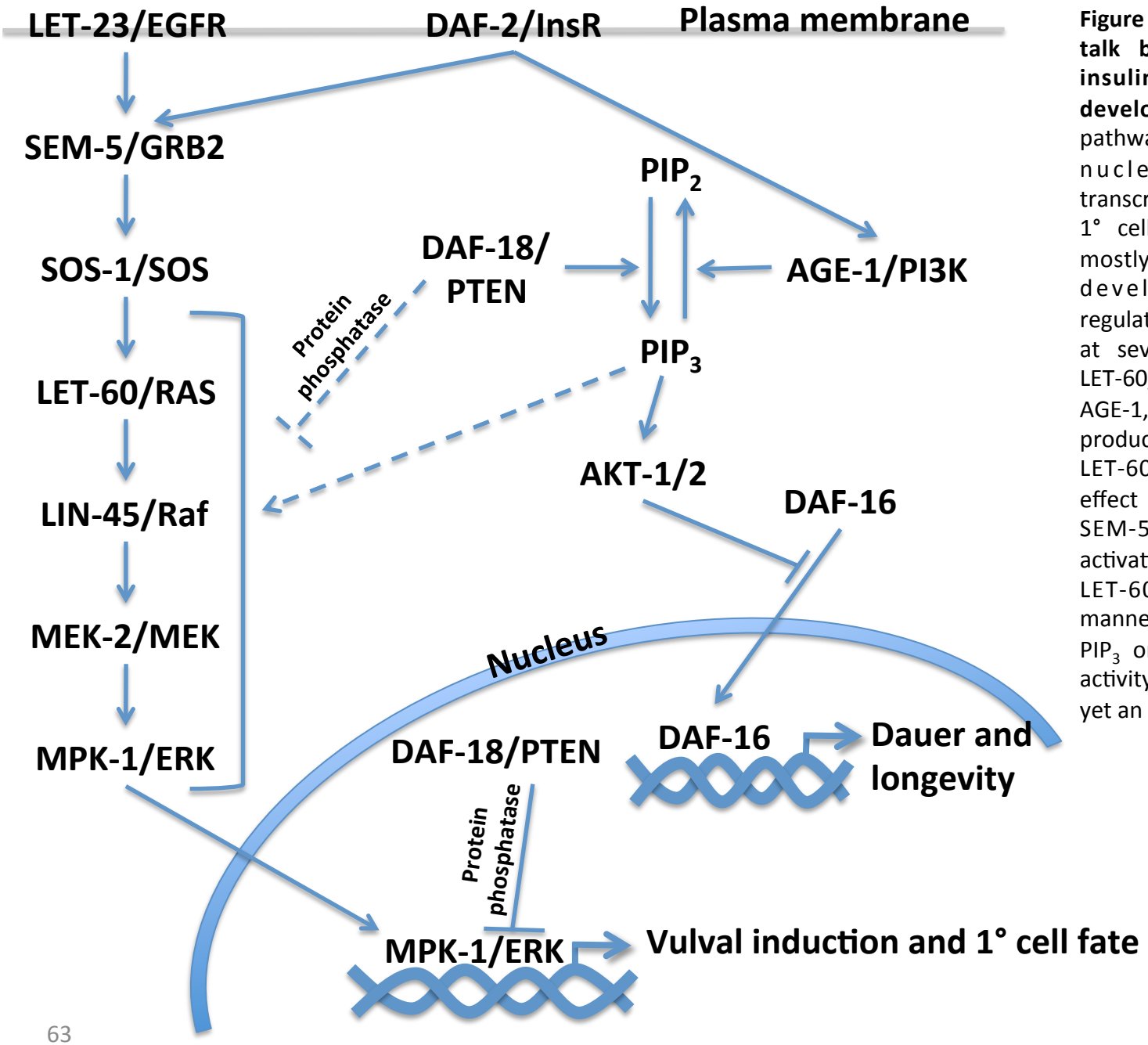


Figure 6. proposed model for cross talk between LET-60/MPK-1 and insulin pathways during vulval development. The LET-60/MPK-1 pathway leads to MPK-1 entering the nucleus where it regulates transcription of genes controlling the 1° cell fate. The insulin pathway, mostly known to regulate dauer development and longevity, regulates the LET-60/MPK-1 pathway at several levels. DAF-2 enhances LET-60/MPK-1signaling by activating AGE-1, which leads to increase in PIP_3 production, which probably enhances LET-60/MPK-1 signaling. Another effect of DAF-2 is recruitment of SEM-5 to the membrane, thus activating LET-60. DAF-18 can inhibit LET-60/MPK-1 signaling in two manners. Either by dephophorylating PIP_3 or via its protein phosphatase activity which dephosphorylates as yet an unknown target.

2.4) Additional experiments not added to the manuscript

Potential substrates of DAF-18 do not affect vulval induction

FAK and SHC have both been previously found to be directly dephosphorylated by PTEN [1, 2]. In order to examine whether these two genes might act as substrates of DAF-18 and thus mediate the protein phosphatase effect of DAF-18 on vulval development, *kin-32/FAK(lf)* and *shc-1/SHC(lf)* mutations were crossed to *daf-18(lf) let-60(gf)* background and vulval induction was quantified. Neither *shc-1* nor *kin-32* had any effect on vulval induction (Table 1), thus they do not appear to mediate the effect of DAF-18 on vulval development.

	genotype	induction	% Vul	% Muv	n
1	*daf-18(lf) let-60(gf)*	4.99±0.81	0	96.4	221
2	*kin-32(lf); daf-18(lf) let-60(gf)*	5.03±1.00	0	93.3	15
3	*shc-1(lf); daf-18(lf) let-60(gf)*	4.97±0.82	0	100	17

Table 1. Epistasis analysis of potential DAF-18 substrates. Induction indicates the average number of induced VPCs per animal. % Vul indicates the fraction of animals with less than three induced VPCs. % Muv indicates the fraction of animals with more than three induced VPCs. Alleles used: LG I: *shc-1(ok198), kin-32(ok166)*, LG IV: *daf-18(ok480), let-60(n1046)*

DAF-18::GFP is expressed in tissues surrounding the vulva

The translational reporter of the genomic *daf-18* was expressed in all VPCs during vulval development, but also in the surrounding tissues such as the Distal Tip Cells (DTCs) (Figure 1A), the Ventral Neuron Cord (VNC) and the uterus (Figure 1B) as well as the occasional expression in the Anchor Cell (AC) (Figure 1C and D). the role of DAF-18 in these tissues remains largely uncharacterized.

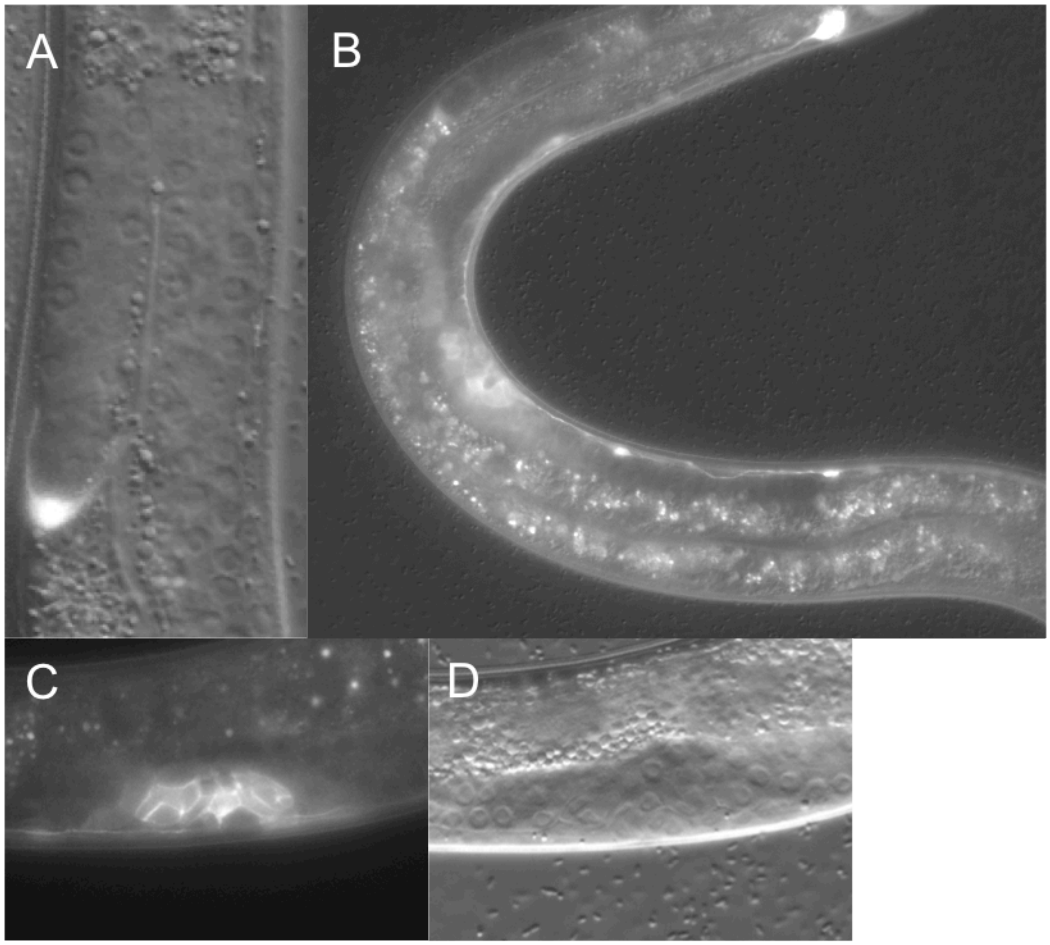


Figure 1. Translational reporter of genomic DAF-18. DAF-18 expressed in different tissues, among them the distal tip cells (A), the ventral neuron cord (B) and on occasion the anchor cell (C and D).

Neuronal expression of DAF-18 partially inhibits vulval induction

The DAF-18 translational reporter was prominently expressed in the Ventral Nerve Cord (VNC). Expression of genes in the VNC has been previously shown to affect vulval induction [3]. In order to examine whether neuronal expression of DAF-18 might rescue the vulval phenotype, the pan-neuronal promoter of *unc-119* was fused 5' of *daf-18 cDNA::gfp::unc-54 3'UTR*, expressed in *let-23(rf); daf-18(lf)* animals and vulval induction was quantified. Although a partial rescue was observed (Figure 2), this result must be taken with great caution, since as previously observed in our laboratory, *Punc-119* also drove expression of DAF-18::GFP in the VPCs.

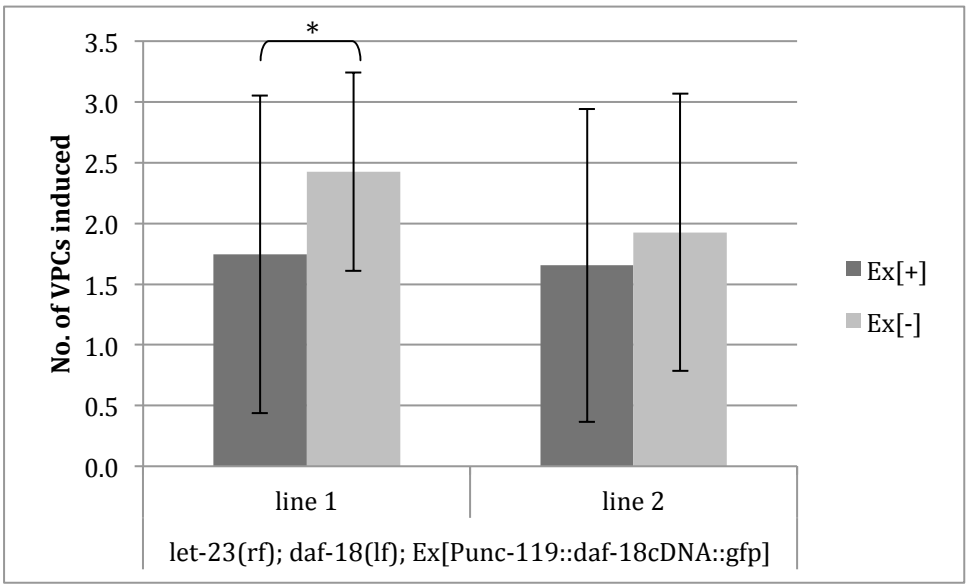


Figure 2. *let-23(rf); daf-18(lf)* animals expressing *Ex[Punc-119::daf-18cDNA::gfp]* were allowed to grow till the L4 stage and vulval induction was quantified using Nomarski imaging. * indicates a p-value of ≤0.05. For each line the t-test was performed comparing the animals with and without the array from the same plate. n≥20 in all experiments. Alleles used: LG II: *let-23(sy1)*, LG IV: *daf-18(ok480).*

LET-60 12V 40C activates LIN-45 in the vulva

Mammalian RAS has three major downstream effectors, being RAF, RalGDS and PI3K. In the worm, LIN-45/RAF has long been considered the main, if not the only downstream effector of LET-60 in the vulva, which positively induces the primary cell fate. Zand et al., however, found that LET-60 also acts in the secondary cells to drive the cells to adopt the 2° cell fate via the RAL-1/RalGDS pathway [4]. As part of their work, they constructed different *let-60(gf)* mutant plasmids containing mutations that preferentially bind only one of the effectors. Upon my request, I received pVG3(*Plin-31::let-60 12V 40C::unc-54 3'UTR*) that preferentially binds and activates AGE-1. Wild-type animals expressing the construct displayed a very strong Muv phenotype. However, the construct also caused a very strong Muv phenotype in an *age-1(lf)* background. Thus, unlike in cell culture, this mutation in the worm appears to be very leaky and to act also via LIN-45.

References:

1. Schneider, E., et al., *Migration of renal tumor cells depends on dephosphorylation of Shc by PTEN.* Int J Oncol, 2011. **38**(3): p. 823-31.
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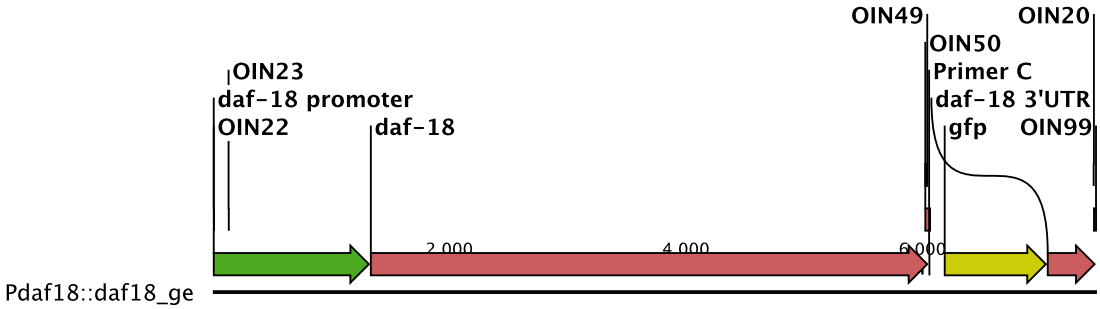
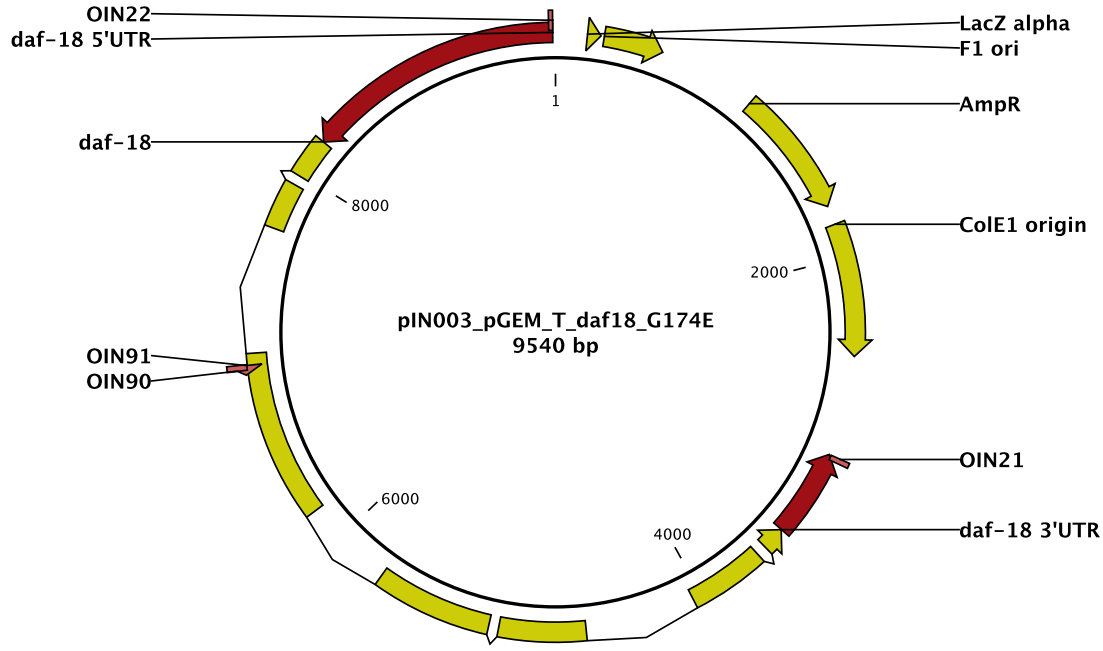
2.5) Main constructs and primer used

For the fully detailed list see Filemaker database from our laboratory.

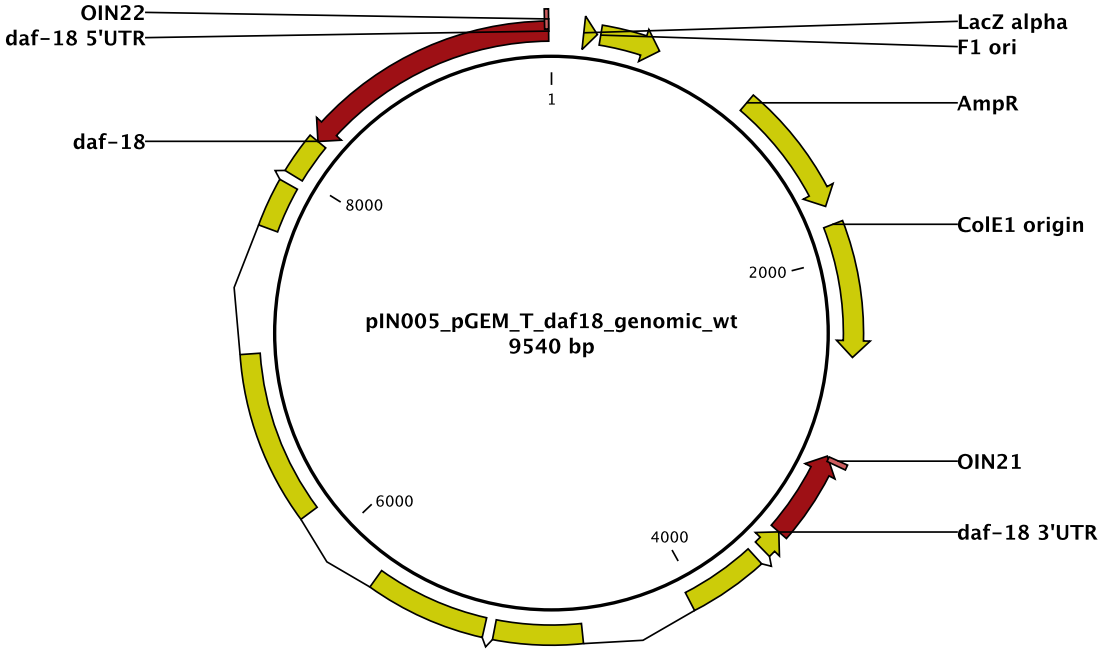
Fusion PCR to make translational reporter of *genomic daf-18::gfp*



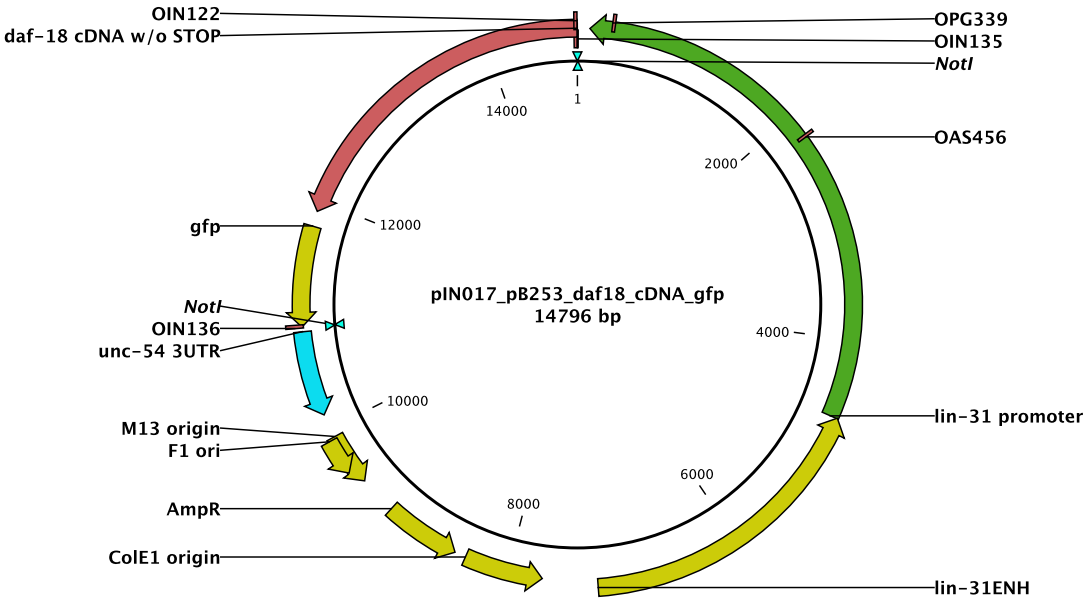
Fusion PCR to make *genomic daf-18 G174E* and cloning to pGEM-T



Cloning *genomic daf-18* to pGEM-T



Cloning *daf-18cDNA::gfp* to pB253 to make *Plin-31::daf-18cDNA::gfp::unc-54 3'UTR*



Primer name	Sequence
IN20	AGATTCTATGTATCGGAGAA
IN21	AAAACAATGTAAATTCGAATGCGCC
IN22	CGTCGATTTTTCCGACTTGT
IN23	ACAACTTCCTCTGGTGGTCC
OIN49	CAAATAAATAGCTTGATCAAAATTCGAATCCG
OIN50	CGGATTCGAATTTTGATCAAGCTATTTATTTGAGCTTGCATGCCTGCAGGTCGACT
OIN90	CAT CAC TCC GGT ACG TTC TTT TCC AGC
OIN91	GA AAA GAA CGT ACC GGA GTG ATG ATA TGT GCT CTT CTC ATC TAC ATC
OIN99	ATAATAAAGTTGGCGCCTAGATTCTATGTATCG
OIN122	ATGGTTACTCCTCCTCCAGATGTGCC
OIN135	TTTGCGGCCGCATGGTTACTCCTCCTCCAGATGTGCC
OIN136	TTTGCGGCCGCGCTATTTGTATAGTTCATCC

2.6) Further discussion and future experiments

The following is further discussion of issues not addressed in the discussion of the manuscript.

The effect of DAF-18 on vulval development appears to be both dependent and independent of AGE-1. I have proposed that the AGE-1-dependent part is via its lipid phosphatase activity, while the AGE-1-independent part is via DAF-18 protein phosphatase activity. To further examine this idea, one could express DAF-18 G174E in an *age-1(lf); daf-18(lf) let-60(gf)* background. Since *age-1(lf)* brings the induction of *daf-18(lf) let-60(gf)* only partially down and DAF-18 G174E does the same, the combination of both might give a full rescue, bringing induction level back to the level of the single *let-60(gf)* mutants. If so, this will be further proof that the protein phosphatase activity of DAF-18 is independent of its lipid phosphatase activity and complements AGE-1 function.

Examining a translational reporter of the genomic DAF-18, I have found the sub-cellular localization of DAF-18 to change during vulval development. The membrane staining observed at the later stages of vulval development was expected and fits the function of DAF-18 as a lipid phosphatase. However, the nuclear localization at the earlier stages of vulval development was less expected and might warrant further examination. One hypothesis is that PTEN acts in the nucleus as a protein phosphatase. To examine this possibility, one could add an NLS signal to either the wild-type DAF-18 or DAF-18 G174E and examine its effect on vulval development. In a similar case, the MPK-1 phosphatase LIP-1 was expressed with an NLS, a modification that led to a dominant Vul phenotype due to suppression of MPK-1. If DAF-18 acts as a protein phosphatase in the nucleus, DAF-18 G174E should have an effect on vulva development. However, if it is the lipid phosphatase that is important in the nucleus, DAF-18 wt should rescue the vulval phenotype.

Another interesting observation was the role of the insulin receptor DAF-2 and the PI3K AGE-1 in vulval development. *daf-2(rf)* suppressed the *let-60(gf)* Muv phenotype nearly to wild-type levels, which appears to be an even stronger effect than *let-23(lf)* [1]. A putative null *daf-2* allele exists which is viable when

combined with *daf-16* deficiency. This strain was ordered and will be examined for its vulval induction. If DAF-2 is *necessary* for vulval induction, we expect to see an induction defect, but if DAF-2 is merely a *modulator*, a vulval phenotype will only be observed in a sensitized background [such as *let-60(gf)*]. Regardless of the result, cell-autonomy of DAF-2 is also an interesting issue to be examined, as DAF-2 is known to act cell-non-autonomously in the dauer pathway and in lifespan. Driving expression of DAF-2 using the *lin-31* promoter in a *daf-2(rf); let-60(gf)* background should help answer this question. Further downstream components of the pathway would then need to be investigated, and the obvious candidate is the PI3K AGE-1. In this work I have found AGE-1 to also be involved in vulval development, although *age-1(lf)* did not suppress *let-60(gf)* Muv as strong as *daf-2(rf)* did. One tempting model would be that LET-60 acts via LIN-45 when it is activated by LET-23, while when activated by DAF-2 it preferentially binds AGE-1 to promote vulval development. To examine this, a quadruple mutant of *daf-16(lf); age-1(lf); daf-2(rf); let-60(gf)* mutant needs to be constructed to examine whether *daf-2(rf)* can further suppress *daf-16(lf); age-1(lf); let-60(gf).* Since I have already made the strain *daf-16(lf); age-1(lf); let-60(gf)*, and since *daf-2* RNAi was shown to work [2], one could try an initial RNAi experiment. As with DAF-2, cell autonomy can be examined by driving *age-1* expression under the control of the *lin-31*promoter.

Another interesting issue regarding DAF-2 activity is its ligand and the source of the ligand. About 40 different insulin ligands have been described in *C. elegans*, with some activating the insulin pathway and some suppressing it. With so many different ligands, one would expect the body of the worm to be systemically loaded with ligands, yet the local concentration of specific ligands is probably important in the different tissues. Gonad ablation affects life span, where ablation of only the germline precursor cells Z2 and Z3 extends life span by up to 60%, an extension that requires the somatic gonad, as ablation of Z1 and Z4 eliminates the life span extension [3, 4]. Gonad ablation also causes a strong Vulvaless phenotype, even in a *let-60(gf)* background [1], which gives an induction of 1.3. If ablating the gonad in a *daf-2(rf); let-60(gf)* would also give an induction of 1.3 that might indicate that a ligand originating from the gonad activates DAF-2 to regulate vulval development.

In conclusion, the insulin and RAS/MAPK pathways appear to crosstalk during vulval development. As both pathways regulate diverse processes, one can assume the insulin pathway is used to transduce environmental signals to the RAS/MAPK pathway to regulate development of many tissues and processes. For example, the RAS/MAPK pathway negatively regulates exit from pachytene of the germline, and positively regulates oocyte maturation. One could see how it would be necessary to sense the environment to affect these processes. If no food is left, the worm will "want" to stop producing new eggs (stop exit from pachytene) and finish quickly the production of already existing ones. In this manner, having the insulin pathway positively regulating the RAS/MAPK pathway could make an evolutionary sense in linking the two pathways. Regarding vulval development, no obvious advantage appears to exist for the two pathways to crosstalk and a possible explanation can be simply that the two pathways remained linked also in this tissue as a byproduct.

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3) Food quality affects vulval development

3.1) Manuscript draft: Food quality rather than quantity regulates EGFR/RAS signaling during C. elegans vulval development

Food quality rather than quantity regulates EGFR/RAS signaling during *C. elegans* vulval development

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Running title: Food quality regulates RAS signaling

Abstract

The relationship between nutrition and proper development and health of an organism is well established. Infants suffering from malnutrition often have problems in development, and mouse models have shown that nutrition affects cancer development and progression. In this work we have used *C. elegans* vulval induction as a model to quantify the output of RAS/MAPK signaling in the whole animal, and examined how food quality can affect RAS/MAPK signaling. We found that the *Comamonas* bacteria strain DA1877, which is considered more nutritious for *C. elegans* than the commonly used *E. coli* strain OP50, caused an increase in RAS/MAPK signaling. We further observed that the increase in RAS/MAPK signaling in animals fed with DA1877 is independent of the insulin pathway, which plays a key role in transducing food signals to evoke specific responses in metazoans. It is thus possible that a new, so far undefined pathway may be a major mediator between nutrition and RAS/MAPK signaling.

Introduction

Environmental conditions greatly affect many aspects of development and adult life in all metazoans. Nutrition, for example, has been implicated in most aspects of development and health, with a growing emphasis on how food can influence the probability of developing cancer as well as influence life span. The first observation linking cancer development and caloric restriction was reported more than a hundred years ago, when Moreschi observed already in 1909 that tumors transplanted in underfed mice did not grow as well as in well fed mice. Currently, it is well established that food quality and quantity can greatly influence the chance of developing cancer, of overcoming carcinogenic elements and even extend adult life span [1]. Dietary factors are thought to account for about 30% of cancer cases in developed countries [2].

C. elegans is a free-living nematode that probably encounters very limited amounts of food resources in the wild. In fact, it is postulated that the worm spends most of its time in the dauer stage. In the laboratory, however, *C. elegans* is grown under optimal conditions on Petri dishes containing NGM media with an abundance of bacteria, which the worm feeds on. The most commonly used strain of bacteria is the *E. coli* strain OP50, which requires external supply of Uracil for efficient growth. NGM media, however, has limited amounts of Uracil, thus the growth of OP50 is limited and the bacteria do not overgrow on the plate. Having a thin layer of bacterial lawn allows better observation of the worms on the plates [3]. However, the amount of bacteria on the plate does not seem to be a limiting factor for the growth of the worm, as the worms do not enter dauer and often have sufficient food for several days before the worms have eaten the entire bacterial lawn. Having ample amounts of food does not necessarily mean having enough nutritional substances, and an indication to this comes from the fact that worms grown on OP50 do not rest, but rather constantly feed and search for food, while worms grown on *E. coli* HB101 or the bacterial specie *Comamonas* were observed to stop feeding and moving and enter a quiescence-like state [4]. This suggests that worms grown on OP50, despite having sufficient amount of bacteria to feed on, might still be exposed to caloric restriction. OP50 is not the only type of bacteria used in laboratories to grow *C. elegans* on. HT115,

for example, is commonly used for RNAi experiments [5] and other *E. coli* strains have been used over the years as well as non-*E. coli* bacterial strains. Avery and Shtonda analyzed how different bacteria affect the growth rate of the worm, in correlation with the size of the individual bacterium [6]. They found that the efficiency of feeding is limited by the number of bacteria the worm can swallow in each pumping of the pharynx and that this number in turn correlates with the growth rate. However, two *E. coli* strains of similar size did not support growth at similar levels, and the authors suggest a possible effect downstream of the actual pumping. Interestingly, out of 15 strains examined, the first six most "nutritious" were not *E. coli* strains, and the second best specie was *Comamonas* sp., which is of importance in this work. Another recent publication showed body size to be dependent on the type of food and the insulin pathway, where wild-type worms grown on the *E. coli* strain HB101 (ranked 7th in the aforementioned publication) were bigger than when grown on OP50. This effect was reversed by mutation of the insulin receptor DAF-2 [7]. Thus in this case food quality affected body size in an insulin pathway dependent manner.

In humans, the EGFR/RAS signaling pathway is mostly involved in cancer development and progression, and activating RAS mutations occur in approximately 30% or all types of cancer [8]. This pathway is highly conserved in *C. elegans*, with many of the components having clear and highly conserved orthologs to human genes [9]. In mammalian cell culture, the RAS/MAPK pathway is often regarded to as an oncogenic pathway regulating cell proliferation [8]. However, evidence exists to suggest the RAS/MAPK pathway is also involved in different developmental diseases (reviewed in [10] and [11]).

In *C. elegans*, the orthologs of EGFR and RAS, termed LET-23 and LET-60, respectively, regulate different developmental processes such as cell fate specification rather than proliferation. The LET-23/LET-60 signaling pathway was found to be active in many tissues, regulating many developmental processes (reviewed by Sternberg and Han [12]). One of the most extensively studied is the development of the egg laying organ of the hermaphrodite, the vulva, where LET-23/LET-60 signaling plays a crucial rule in specifying the primary cell fate. Vulva development begins during the second larval stage, when a specialized cell in the somatic gonad, called the Anchor Cell (AC) starts

releasing the EGF ligand LIN-3. Six Pn.p cells (P3.p-P8.p) termed Vulva Precursor Cells (VPCs), aligned along the ventral side of the worm, all have the potential to receive this signal and to be induced to create the vulva. In a wild-type worm, however, only three cells (P5.p-P7.p) are induced and divide three times to create the adult vulva. The remaining cells are un-induced, divide once, and fuse to the surrounding hypodermis. In a situation where the LET-23/LET-60 pathway becomes hyper-activated, more than three cells get induced, a phenotype called Multivulva (Muv). Accordingly, if the LET-23/LET-60 pathway is hypo-activated, fewer than three cells get induced, a phenotype called Vulvaless (Vul). By counting the number of VPCs induced, one can quantify the level of activity of the LET-23/LET-60 pathway in the worm.

In this work, we have used the vulva induction index to examine how different bacteria, correlating with different food quality, affect LET-23/LET-60 signaling. We have found that the bacterial strain DA1877, which is considered more nutritious than OP50, enhances LET-23/LET-60 signaling in a manner that is independent of the insulin pathway. Thus there exist other possibilities besides insulin, by which the worm senses the environment and nutritional status, suggesting new pathways by which food might affect cancer development in humans.

Results

Food quality, rather than food quantity, affects vulval development

In order to examine whether different quality of bacteria can affect vulval development, animals were grown on either standard OP50 or the more nutritious DA1877 bacteria. While wild-type animals showed no change in vulval induction (data not shown), *let-60(gf)* worms grown on the bacterial lawn of DA1877 had a significantly higher induction (i) compared to those grown on normal OP50 (i=4.93±0.71, n=41 and i=4.09±0.77, n=46, respectively, p-value ≤0.0005) (Figure 1A). Since DA1877 is considered to be "richer", we wondered whether differences in the amount of food the worm eat might cause the increase in induction. In order to examine this, the OD of the bacteria was measured prior to seeding and concentrations of 1x and 12xOP50 were used (see materials and methods). No difference in induction level was observed due to increased concentration of the bacteria (i=3.58±0.75, n=18 and i=3.61±0.60, n=17, respectively)(Figure 1B), suggesting that indeed food quality rather than quantity is the cause for the increased induction in the *let-60(gf)* background.

Increased induction on rich food is a general phenomenon

let-60(n1046gf) vulval induction is known to be especially sensitive to changes in the environment and to accumulation of suppressors (personal communication). We therefore examined the more stable and reproducible *let-23(sy1rf)* allele. Although the single mutant was unaffected by the rich food (i=0.55±0.92, n>50 on OP50 and i=0.71±1.07, n=17 on DA1877), when combined with *daf-18(lf)*, which leads to higher induction and thus a more sensitized background, worms grown on DA1877 bacteria had an even higher induction (2.02±1.27, n=43 on OP50 and 2.56±0.86, n=40 on DA1877, p-value ≤0.05)(Figure 1A). Thus, the increase in vulval induction in worms grown on rich food is not allele specific but rather a general phenomenon, though more alleles need to be tested.

Increased induction on rich food is independent of the Insulin pathway

Since the insulin pathway is crucial in sensing the environment and affects development accordingly and since insulin signaling has been recently shown to play a role in vulval induction (I. Nakdimon, A. Hajnal, manuscript draft, see chapter 2.3), we examined whether mutations in the insulin pathway would also react to the different food sources. *daf-2(rf); let-60(gf)* animals were grown on 1xOP50, 12xOP50 and 1xDA1877 plates and the number of cells induced was counted. Again, higher concentration of OP50 had no effect on vulva induction (i=3.32±0.77, n=19 and i=3.06±0.25, n=16, respectively), while worms grown on DA1877 showed a significant increase in induction (i=4.03±0.87, n=19, p-value≤0.05 compared to 1xOP50). Similar results were obtained for *age-1(lf); daf-18(lf) let-60(gf)* animals (4.62±0.92, n=20 on OP50 and 5.17±0.69, n=20 on DA1877, p-value≤0.05)(Figure 2). Thus, animals defective in the insulin pathway do exhibit increased vulval induction when fed on DA1877 bacteria, suggesting that an alternative pathway is involved in sensing the food quality.

Increased induction on rich food is not inherited

In order to examine whether the increase in vulval induction is due to epigenetic modifications that can be inherited, *let-60(gf)* mutants grown on DA1877 were treated with sodium hypochlorite solution to release the eggs and the offspring were allowed to hatch in buffer without bacteria for one day, at which time they were transferred to either OP50 plates or DA1877 plates. While worms transferred to plates with DA1877 bacteria continued to display increased induction, worms grown on OP50 reverted to the normal *let-60(gf)* induction level (i=4.9±0.81, n=10 on DA1877 and i=4.14±0.59, n=18 on OP50, p-value≤0.05)(Figure 3). This result suggests that epigenetic modifications do not cause the increased induction on rich food.

Other E. coli strains also affect vulval induction

OP50 is a strain of E. coli commonly used in *C. elegans* laboratories as standard food for the worms. DA1877 however, is not an E. coli strain, but rather belongs to the *Comamonas* sp.. To examine whether other strains of E. coli might affect vulval development, *let-60(gf)* worms were grown on either BL21 or HT115 E. coli strains (Data of Figure 4 will be added to the final version).

Rich food increases RAS/MAPK signaling in the VPCs

Increased induction in the *let-60(gf)* background often indicates increased RAS/MAPK signaling. To examine if that is indeed the case, *let-60(gf)* worms carrying the integrated EGL-17::CFP transcriptional reporter, which is commonly used to quantify LET-60 signaling in the VPCs at the early stages of vulval development (2-4 cells stages), were grown on either DA1877 or OP50 bacteria, and EGL-17::CFP fluorescence levels were quantified at the 2 and 4 cells stages of vulval development. EGL-17::CFP expression levels were significantly increased in the secondary descendants of P5.p and P7.p in worms grown on DA1877 compared to worms grown on OP50 (mean fluorescence was 489±524 vs. 19±184 in P5.p, 250±344 vs. 15±232 in P7.p, n=18 and n=24 respectively, p-value≤0.005 and p-value≤0.05 respectively) (Figure 5). Interestingly, EGL-17::CFP levels in P6.p appeared to be slightly decreased in worms grown on DA1877 compared to worms grown on OP50 (1352±653 vs. 1658±617, n=18 and n=26, respectively, p-value=0.13)(Figure 5). Although the difference was not statistically significant, it was consistent at both the 2 and 4 cells stages. Finally, EGL-17::CFP expression levels are often indicative of the cell fate, where high levels of expression during the 2-4 cell stages correlate with the cell adopting a primary cell fate. When comparing EGL-17::CFP expression levels between secondary and primary cells (2°/1°), none of the 24 *let-60(gf)* animals grown on OP50 had a ratio above 0.4 of 2°/1° (Figure 6A) while in animals grown on DA1877, 5 out of the 18 examined animals had a 2°/1° ratio of EGL-17::CFP expression above 0.4 (Figure 6B), indicating a possible cell fate change caused by DA1877 bacteria.

LET-23::GFP expression pattern does not change on DA1877

The elevated in EGL-17::CFP expression in the secondary cells and the slight decrease in P6.p suggests a potential sequestering effect of the LIN-3 signaling similar to the model proposed by Hajnal et al. [13]. Perhaps in this case, food quality affects membrane composition, which leads to increased LET-23 levels at the baso-lateral side of the membrane in the secondary cells, allowing P5.p and P7.p to receive more LIN-3 signal. However, an integrated translational reporter of LET-23::GFP (generated in our lab by Juan Restrepo) did not show any significant difference in localization or expression levels in animals grown on DA1877 compared to OP50 (Figure 7).

LIN-3 level in the anchor cell does not change on DA1877

Another possible explanation for the increased EGL-17::CFP expression in the secondary cells might be increased production of LIN-3 by the AC. However, an integrated LIN-3::GFP transcriptional reporter did not show any change in the level of expression between worms grown on DA1877 compared to OP50 (expression level of 1952±536 and 1994±490 respectively) (Figure 8).

Bacterial size and shape

By examining different bacterial strains, Avery and Shtonda [6] showed a correlation between bacteria size and nutritional value, where smaller bacteria could better support growth. Examination of OP50 and DA1877 revealed obvious differences in size and shape of the two bacteria (Figure 9A and B). While OP50 is relatively short and wide (average height of 1.96±0.46μM, width 1.21±0.09μM, ratio of height/width of 1.61), DA1877 is long and narrow with a bend in the middle (average height of 2.85±0.36μM, width 0.88±0.11μM, ratio of height/width of 3.25) (Figure 9C). Assuming a cylindrical shape of the bacteria, the average volume of OP50 is 2.31±0.69μM, while DA1877 had a smaller volume of 1.76±0.58μM. How this characteristics of the bacteria might affect the RAS/MAPK signaling in worm is of ongoing research as discussed below.

Discussion

The quantity and quality of what we eat affects our development and health. Better understanding of the molecular pathways involved in these processes will allow us to find treatment for diseases caused by malnutrition, such as cancer and obesity and eventually extend healthy life span. Using *C. elegans* vulval development to quantify RAS signaling, we found that growing the worms on the more nutritional bacteria, *Comamonas* DA1877, led to increased signaling of the mitogenic EGFR/RAS signaling cascade, as observed by an increase in the number of VPCs induced. Surprisingly, this increase was independent of the canonical insulin pathway. One possible reason for the increase in EGFR/RAS signaling may simply be larger amount of food being ingested by the pharynx, which would then activate a further downstream signal to enhance the EGFR/RAS pathway. However, when worms were grown on concentrated OP50, which should allow more food to be pumped, no enhancement in vulval induction was observed. Furthermore, growing worms on bacteria that do not properly support growth leads to an increase in the rate of pharyngeal pumping, while worms grown on bacteria that better support growth, leads to a decrease in the rate of pharyngeal pumping [14]. Thus, the worm is probably able to compensate for insufficient nutritional food by increasing the rate of food ingested, which increases the probability of a qualitative difference between the bacteria we compared. Finally, examining the shape of the bacteria suggests that OP50 may be more easily pumped, as they are relatively round and uniform in shape while DA1877 have a slight bend in the center, which could prevent the bacteria from packing in a tight manner for efficient consumption. On the other hand, the shape of DA1877 seems to be more fragile compared to OP50, which may allow easier grinding of the bacteria to extract the nutrients.

Beyond the simple increase in vulval induction, also expression of the 1° marker EGL-17::CFP increased in worms grown on DA1877. *egl-17* expression is regulated by RAS/MAPK signaling in primary cells at the 1-4 cells stages, and is commonly used as a primary cell fate marker [15]. The slight decrease in EGL-17::CFP in P6.p may very well be a side effect of the increase in RAS/MAPK signaling in the 2° cells. Increased RAS/MAPK signaling in the 2° cells also results in increased lateral signal coming from the 2° cells to the 1° cell and activation of

the Notch pathway in P6.p. Activation of Notch signaling in P6.p can then lead to inhibition of RAS/MAPK signaling [16]. However, the question remains what caused the increase in RAS/MAPK signaling in the 2° cells. One possible explanation, as previously mentioned, is that DA1877 might cause changes in LET-23 localization such as increased basolateral accumulation in 2° cells. Having more LET-23 in 2° cells can sequester the LIN-3 ligand so that less LIN-3 is available for the 1° cell. Since no obvious change in LET-23::GFP localization was observed another explanation is possible, which is increased LIN-3 production [17]. An increased LIN-3 concentration could saturate LET-23 in P6.p, allowing more RAS/MAPK signaling in the 2° cells, which will then send out the lateral Notch signal back to the 1° cell which will cause a decrease in RAS/MAPK signaling in the 1° cell, despite the higher levels of LIN-3. However, here too, no apparent difference was observed, though the marker used may not be appropriate. However, the AC is not the only source of LIN-3 in the worm. The hypodermis is also known to be able to produce LIN-3, yet under normal conditions it is inhibited. In l*in-15(lf)* mutants this inhibition is removed and an excess of LIN-3 is produced from the surrounding hypodermis [18].

Our work has so far shown a direct link between nutrition and the carcinogenic RAS/MAPK pathway. We are not the first to define such a connection [19], yet using *C. elegans* genetic and molecular tools, one may better define the exact molecular mechanisms and the components in the food that affects RAS/MAPK signaling. Finding the pathways allowing the different nutrients to regulate RAS/MAPK signaling may open the door to new therapeutic interventions in humans.

Materials and methods

General worm methods

Standard methods were used for maintaining and manipulating Caenorhabditis elegans [3]. Animals were cultured at 20°C on NGM plates seeded with either OP50 or DA1877 bacterial lawn. The wild-type strain is the Bristol N2 strain. Information regarding the mutants used in this study can be found on WormBase (http://www.wormbase.org). Mutations used according to their linkage group: LG II: *age-1(mg44), let-23(sy1)*, LG III: *daf-2(e1370)*, LG IV*: let-60(n1046), daf-18(ok480)*.

Integrated transgenic arrays (transgenes; co-transformation marker): *syIs90[egl-17::yfp + unc-119(+)]* [20] , syIs107[*lin-3::gfp + unc-119(+)]* [21]

Using concentrated OP50

In order grow worms on more concentrated media we grew OP50 overnight at 37°C, measured the OD and used it for seeding. We then centrifuged the bacteria, removed most of the supernatant and measured the OD again. We thus got a 12-fold increase in the OD of the bacteria, which were then seeded onto the plates.

Fluorescence microscopy

CFP expression was observed under fluorescent light illumination with either a Leica DMRA microscope equipped with a cooled CCD camera (Hamamatsu ORCA-ER) or Olympus BX61 with Q Imaging Fast 1394 Retiga 2000R camera (Q Imaging Inc., Canada) controlled by the Openlab 5 software (Improvision/PerkinElmer). Animals were mounted on 4% agarose pads in M9 solution with 20mM tetramisole hydrochloride. Quantification of fluorescence levels was performed under the same picture acquisition settings for all conditions examined.

Vulval induction

Vulval induction was scored by examining worms at the L4 stage under Nomarski optics as described [22]. The number of VPCs that had adopted a 1° or 2° Vulval fate was counted for each animal and the induction index was calculated by dividing the number of 1° or 2° induced cells by the number of animals scored. Statistical analysis was performed using a t-test for independent samples.

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Figure Legends

Figure 1. Effect of food quality and quantity on vulval development.

L1 larvae of different genetic backgrounds were transferred to plates seeded with either *E. coli* OP50, *Comamonas* DA1877 or concentrated OP50 (12xOP50) and the number of induced VPCs of the progeny at the L4 stage was quantified using Nomarski imaging. * indicates a p-value ≤0.05, *** indicates a p-value ≤0.0005. For each line the t-test was performed comparing animals grown on OP50 against animals grown on DA1877 or concentrated (12x) OP50.

Figure 2. Food effect on vulval development is independent of the insulin pathway.

let-60(gf) animals carrying mutations in components of the insulin pathway were raised on either OP50, DA1877 or concentrated OP50 (12xOP50) bacteria and vulval induction of their progeny was quantified using Nomarski imaging. * indicates a p-value ≤0.05. For each line the t-test was performed comparing animals grown on OP50 against animals grown on DA1877.

Figure 3. Food effect is not inherited.

let-60(gf) animals were maintained on DA1877 for several generations, adult hermaphrodites where then treated with sodium hypochlorite solution to release the eggs and the offspring were allowed to hatch in buffer without bacteria for one day and the hatched L1 larvae were transferred to plates seeded with either OP50 or DA1877. When the worms reached the L4 stage, vulval induction was quantified using Nomarski imaging. * indicates a p-value ≤0.05. t-test was performed comparing animals grown on OP50 against animals grown on DA1877.

Figure 4. Other *E. coli* strains also affect vulval development.

To be added to final version.

Figure 5. Quantification of EGL-17::CFP levels in the VPCs.

let-60(gf) animals were grown on plates seeded with either OP50 or DA1877 and the expression level of the primary cell fate marker EGL-17::CFP was quantified using Nomarski and fluorescence imaging. Horizontal bold lines indicate average fluorescence level. * indicates a p-value ≤0.05, ** indicates a p-value of ≤0.005. For each VPC t-test was performed comparing animals grown on OP50 against animals grown on DA1877.

Figure 6. Ratio of EGL-17::CFP expression between 2° and 1° cells.

let-60(gf) animals were grown on plates seeded with either OP50 or DA1877 and the expression level of the primary cell fate marker EGL-17::CFP was quantified using Nomarski and fluorescence imaging. Values of EGL-17::CFP for each 2° cell in each animals were compared to the values measured in the same animal in the 1° cell.

Figure 7. LET-23::GFP expression pattern.

Worms expressing an integrated functional LET-23::GFP reporter were grown on plates seeded with either OP50 (A and C) or DA1877 (B and D) and the expression pattern was examined using Nomarski (A and B) and fluorescence (C and D) microscopy.

Figure 8. Quantification of LIN-3::GFP transcriptional reporter levels in the AC.

Worms expressing an integrated transcriptional reporter of LIN-3::GFP were grown on plates seeded with either OP50 or DA1877 and the expression level was quantified using Nomarski and fluorescence imaging. Horizontal bold lines indicate average fluorescence level.

Figure 9. Size and shape of OP50 and DA1877.

OP50 and DA1877 were grown over-night at 37°C in 2xTY medium. A sample OP50 (A) and DA1877 (B) were then examined using Nomarski optics. C) Measurements of the sizes of the bacteria. Numbers are in micrometers ± standard deviation. h/w represents the ratio between the height and width of the bacteria.

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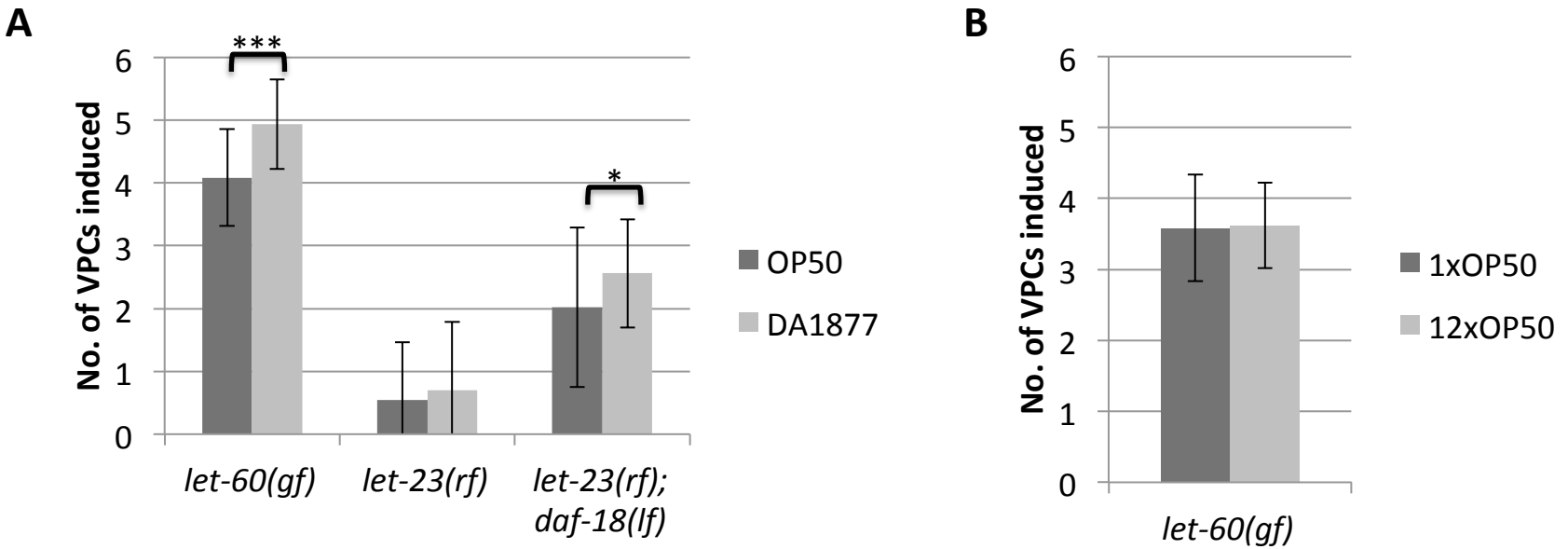

A
No. of VPCs induced
0
1
2
3
4
5
6
***
*
let-60(gf)
let-23(rf)
let-23(rf); daf-18(lf)
OP50
DA1877
B
No. of VPCs induced
0
1
2
3
4
5
6
let-60(gf)
1xOP50
12xOP50

Figure 1

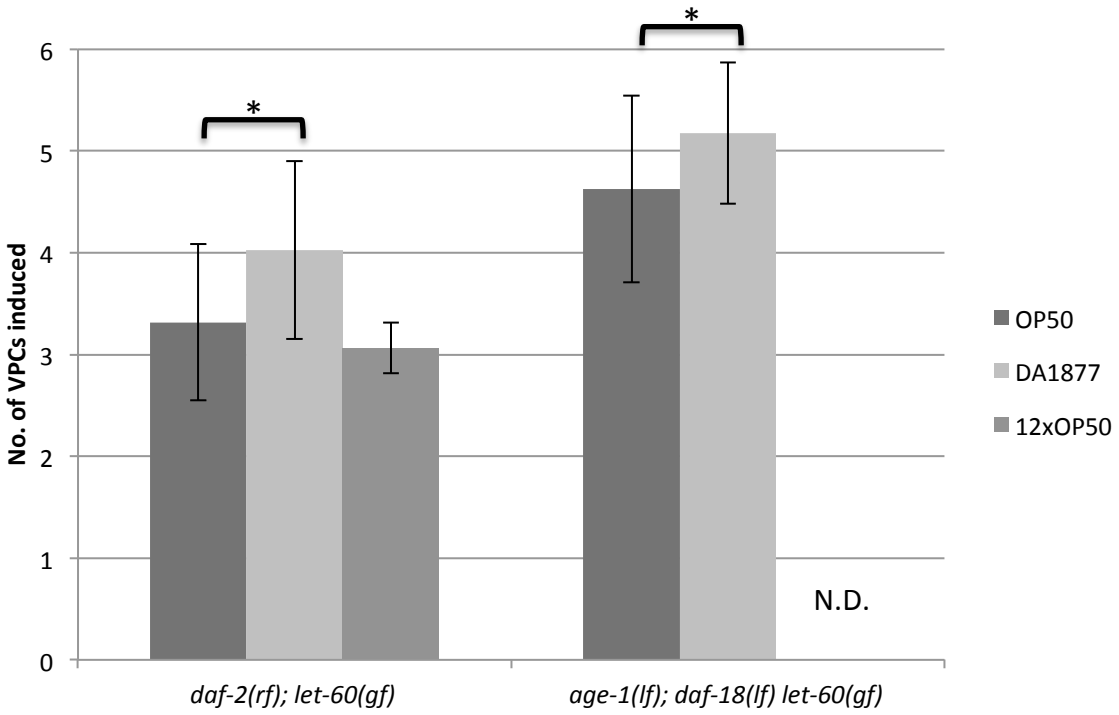

No. of VPCs induced
6
5
4
3
2
1
0
*
*
N.D.
daf-2(rf); let-60(gf)
age-1(lf); daf-18(lf) let-60(gf)
OP50
DA1877
12xOP50

Figure 2

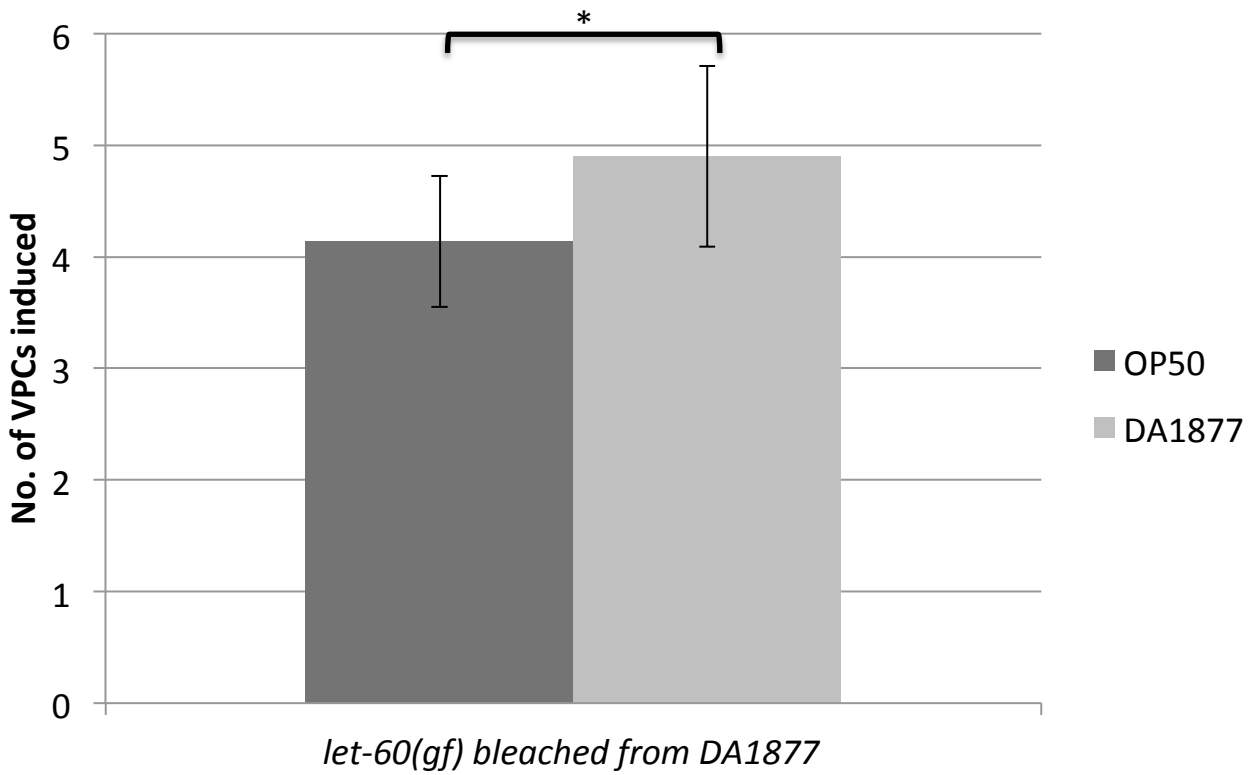

*
6
5
4
3
2
1
0
No. of VPCs induced
OP50
DA1877
let-60(gf) bleached from DA1877

Figure 3

Figure 4

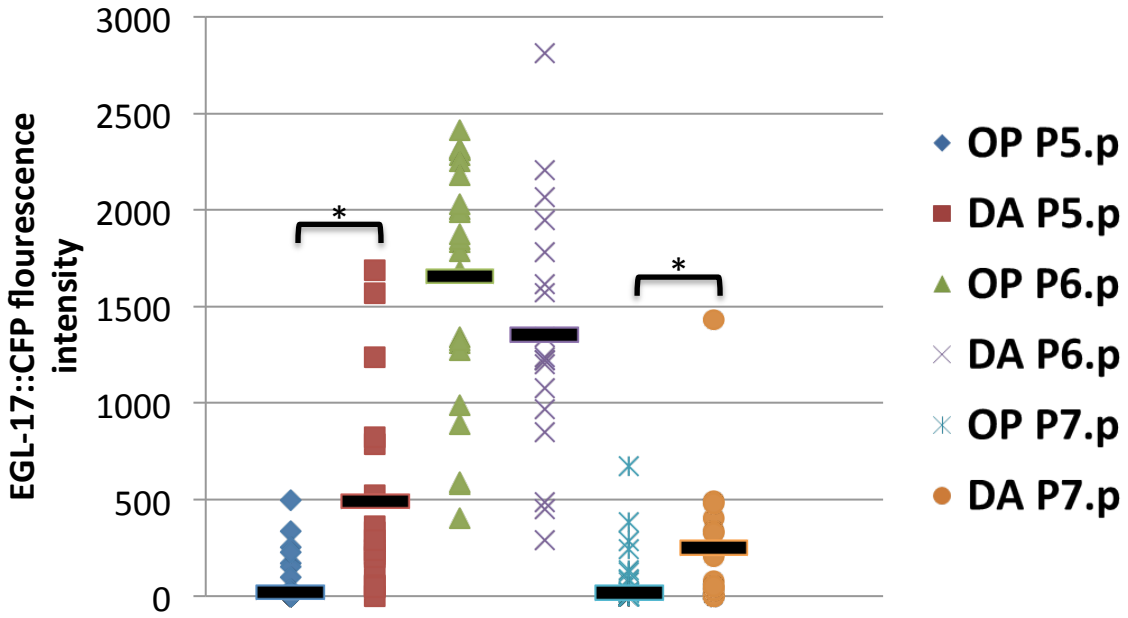

EGL-17::CFP flourescence intensity
3000
2500
2000
1500
1000
500
0
*
*
OP P5.p
DA P5.p
OP P6.p
DA P6.p
OP P7.p
DA P7.p

Figure 5

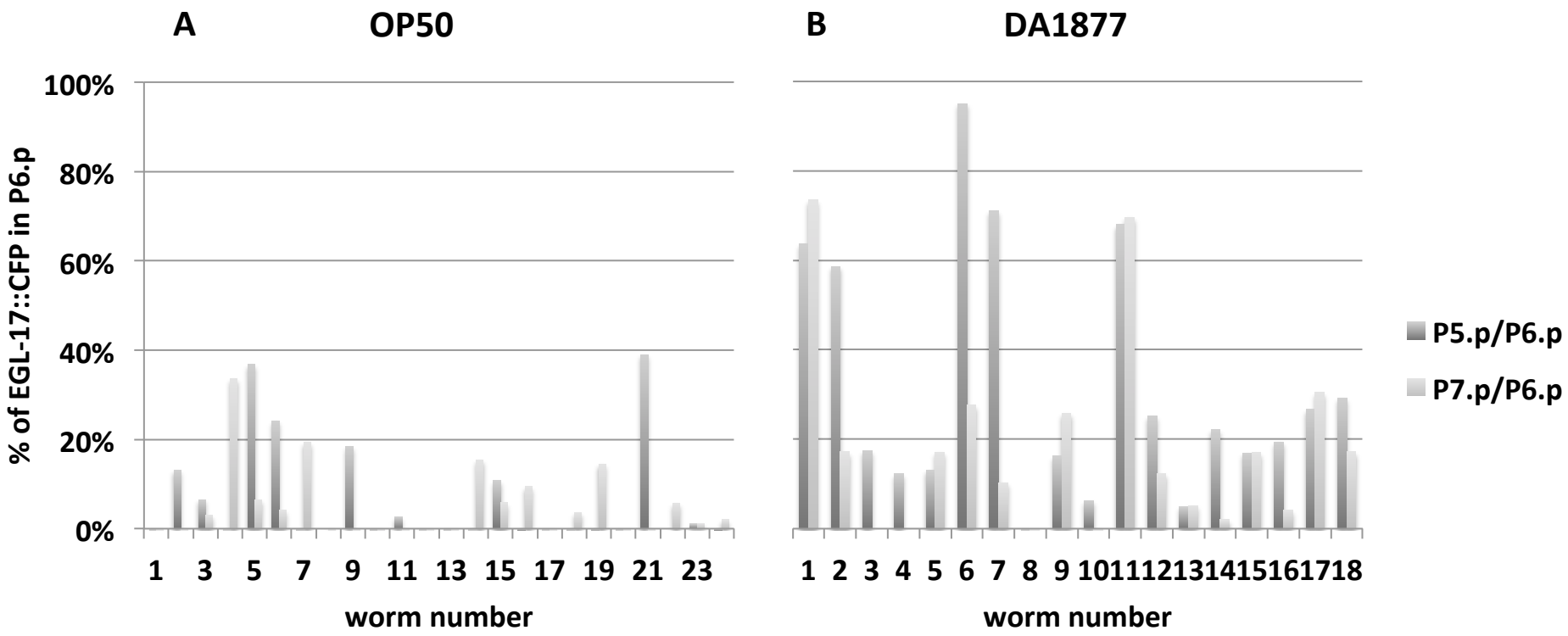

A
OP50
B
DA1877
100%
80%
60%
40%
20%
0%
% of EGL-17::CFP in P6.p
1 3 5 7 9 11 13 15 17 19 21 23
worm number
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
worm number
P5.p/P6.p
P7.p/P6.p

Figure 6

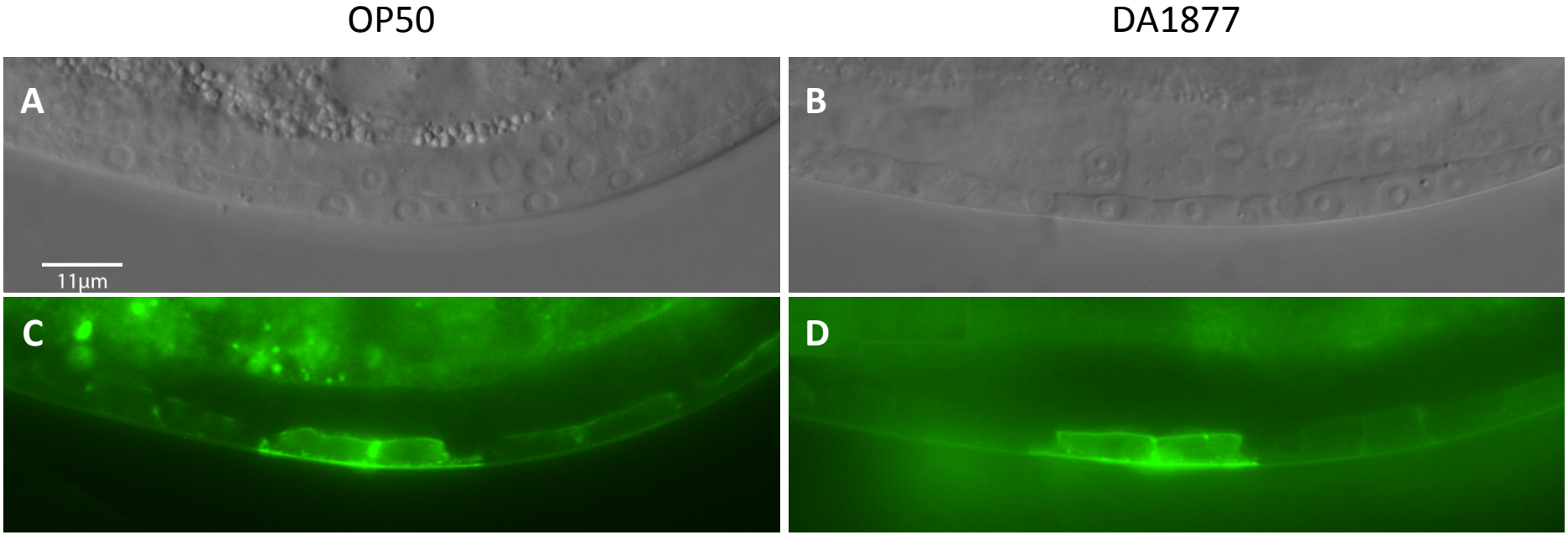


Figure 7

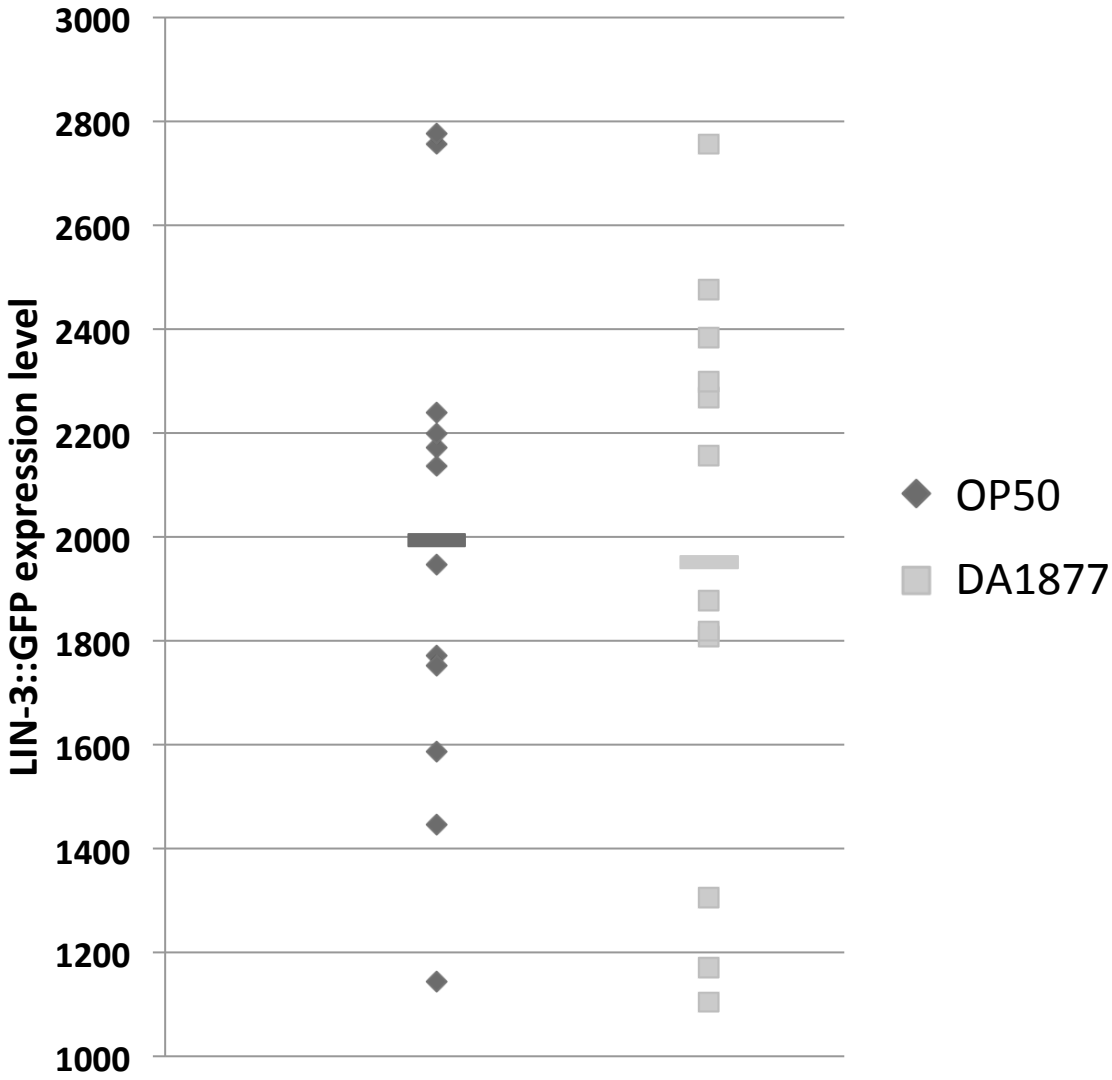
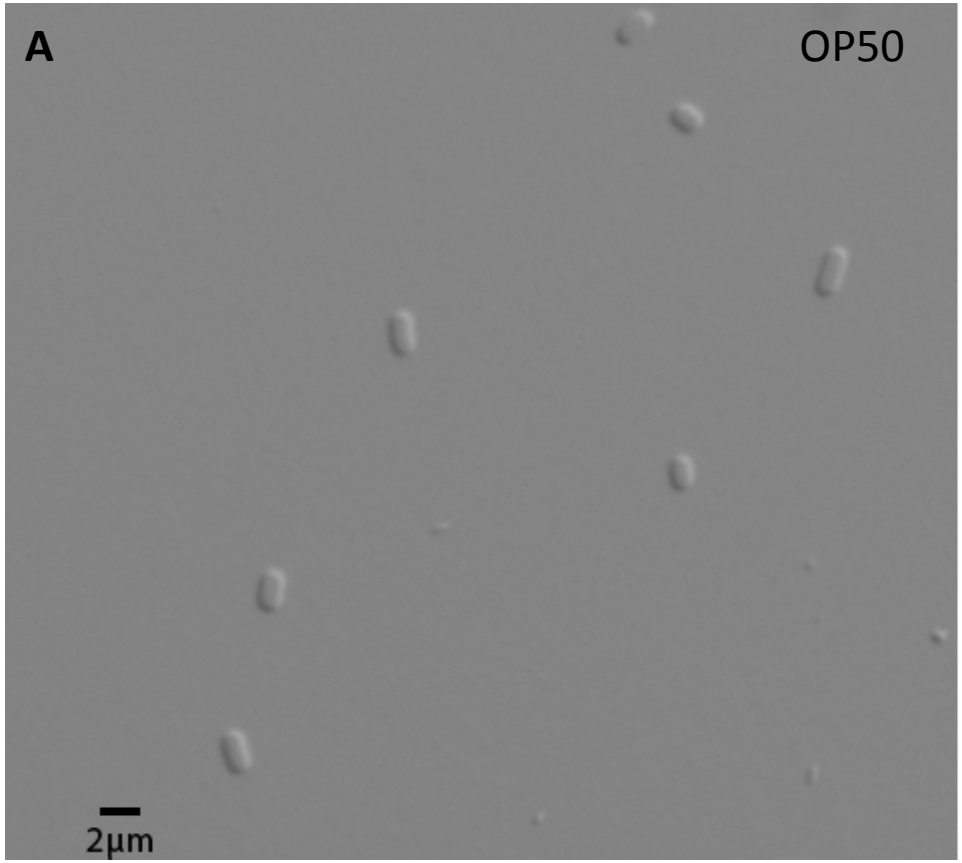
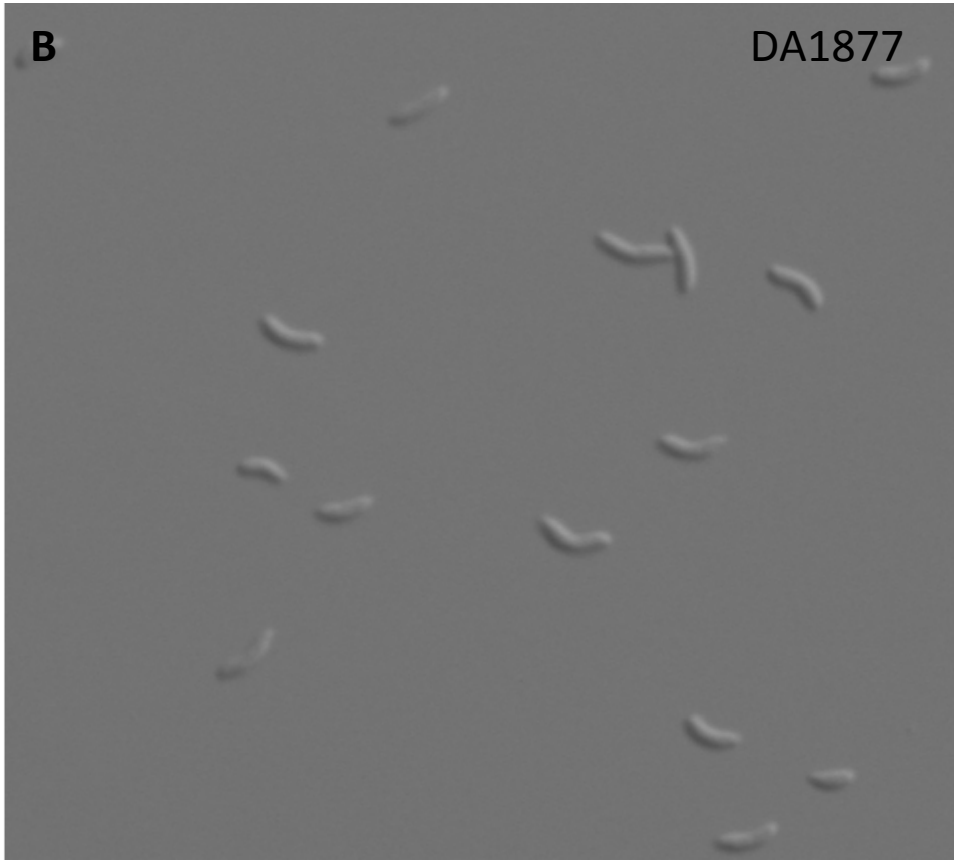

3000
2800
2600
2400
2200
2000
1800
1600
1400
1200
1000
LIN-3::GFP expression level
OP50
DA1877

Figure 8





C

	OP50				DA1877			
	width	height	h/w	volume	width	height	h/w	volume
Avg [uM]	1.21 ± 0.09	1.96 ± 0.46	1.61 ± 0.36	2.31 ± 0.69	0.88 ± 0.11	2.85 ± 0.36	3.25 ± 0.58	1.76 ± 0.58

Figure 9

3.2) Further discussion and future experiments

Many questions remain open in this work. For one, *let-60(gf)* displayed a significant increase in the number of VPCs induced on rich food, but *let-23(rf)* did not, unless combined with *daf-18(lf)*. One explanation for this would be that *let-23(rf)* alone is not sensitive enough to show a phenotype. Signaling pathways seem to expand and increase the further downstream the component is and it is possible that a slight, undetectable increase in signaling at the level of LET-23 can be magnified to a large, phenotypically significant increase at the level of MAPK activity. In order to show that *let-60* is not the exception but rather the rule, other sensitized backgrounds need to be examined, for example a GRB2 *sem-5(lf)* mutant.

Regarding LET-23 localization, although no obvious difference was observed, further examination is still needed. For one, better resolution confocal microscopy and quantification of apical vs. basal intensity ratios may still reveal differences not observed with normal microscopy. Another interesting aspect of LET-23 localization is the dynamics of the receptor. A FRAP protocol has recently been established in our lab, and analyzing recovery or recycling rate of LET-23 on DA1877 compared to OP50 might help explain the increased LET-23/LET-60 signaling.

Another element that warrants further examination is the size and shape of the bacteria. Having a denser bacterial lawn had no effect on vulval induction, yet it is possible that simply condensing the food is not sufficient to allow more food to be ingested with each pumping of the pharynx. In order to examine if it is the quality of the food or quantity, one will need to have the bacteria the same size. For this purpose sonication can be performed that should break down the bacteria to smaller, uniformly sized particles.

Another way to examine if it is the amount of food ingested is by using mutants defective in the rate of pharynx pumping. *eat-2* mutants have a slower rate of pharynx pumping [1]. If it is indeed the amount of food ingested that enhances RAS dependent vulval induction, growing *eat-2(ad465) lin-7(e1413)* mutants (obtained from the CGC) on DA1877 will not cause an increase in vulval

induction compared to worms grown on OP50, while *lin-7(e1413)* single mutants will show an increase.

To examine if the enhancement in vulval induction requires input from the sensory system, the *osm-5(p813)* mutation, which disrupts the activity of the sensory neurons, can be analyzed [2]. If *osm-5(p813); let-60(gf)* double mutants do not display enhancement in vulval induction on DA1877 bacteria compared to OP50, this would mean the input from the sensory system is important for the observed phenotype. If OSM-5 is indeed necessary, based on previous work from our lab, the GPCR SRA-13 might be the component that mediates the signal coming from the sensory system to affect vulval development [3], and should be examined.

Previous publications reported that also different *E. coli* strains had an effect on aging, size and more. I have tried growing worms on other strains such as BL21 and HT101 with conflicting results. At times, there was an obvious difference compared to worms grown on OP50 and at times there was not. Further examination is still needed on the topic. In this regard, it would be interesting to see if there is a correlation between bacterial size or shape and the effect on vulval induction.

Finally, perhaps even a more interesting question is the why. What evolutionary-biological reason is there for food quality to affect the ubiquitously used RAS/MAPK signaling pathway? The answer might be, as the saying goes, too much of a good thing is also not good. As in many biological processes, a balance and control of signaling thresholds is crucial in maintaining proper development of cells, tissues and organisms. Both nutrition and RAS/MAPK signaling are extremely important for healthy development, and linking the two signals makes for valid biological sense. One can easily see the benefit of RAS/MAPK signaling receiving positive feedback from the environment regarding the availability of energy resources. However, when mutations occur, as in the case of *let-60(gf),* the increase in signaling indeed makes an already sick animal even sicker. But, when looking at the other side of the coin, the Vulvaless *let-23(rf); daf-18(lf)* became healthier with regard to vulval development, pointing again to the importance of maintaining a proper balance of signaling. Thus, under normal

conditions the positive feedback of nutritional food is necessary and in fact can contribute to healthy development.

References:

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4) Cell specific mRNA pull down using the FLPase system

4.1) Abstract

Vulval development in *C. elegans* serves as an excellent model to examine complex molecular signaling. Research over the years has helped indentify novel components of the RAS/MAPK and notch pathways and to further characterize the already known ones. However, only a handful of genes have been identified so far to drive the vulval cell to adopt their specified fate. In this work, we used the FLPase system to drive specific expression of the poly A binding protein PAB-1 in the primary cells of the vulva in order to perform cell specific pull down of the mRNAs expressed in these cells during cell fate decision.

4.2) Introduction

4.2.1) Vulval development

During the L2 stage of development, the Anchor Cell (AC), located at the somatic gonad, secrets the EGF like ligand LIN-3. The EGF receptor LET-23, expressed on the basolateral side of P6.p, binds the LIN-3 and activates the Ras/MAPK pathway in P6.p, thus causing the cell to adopt a primary cell fate. In parallel, the Ras/MAPK pathway in P6.p activates the lateral notch signal in the neighboring cells P5.p and P7.p. Activation of the notch pathway in these cells inhibits the Ras/MAPK pathway and causes the cells to adopt a secondary cell fate.

The main components of the two pathways are very well described, yet the specific genes regulated by these pathways that lead to cell fate specification are mostly unknown.

4.2.2) Using PAB-1 to pull down tissue- or cell-specific mRNAs

In *C. elegans*, tissue specific micro-array experiments have previously been successfully performed [1, 2]. In these experiments, a tissue specific promoter was used to express a tagged PolyA Binding Protein (PAB-1) in the tissue of interest. The tagged PAB-1 bound to mRNA was then isolated by

immunoprecipitation, the mRNA was purified, and analyzed using micro-arrays. Performing such an experiment with a vulva specific promoter is currently not possible, since no single promoter is known to be specific enough for the 1° or 2° lineage.

4.2.3) The FLPase system

The FLP recombinase system [3] has recently been successfully used in *C. elegans* by Davis et. al. [4] and Voutev et. al.[5]. In this system, expression of a gene of interest can be temporally and spatially controlled by a technique involving the FLP enzyme, which catalyses recombination between specific target DNA sequences called FRTs sites(FLP Recognition Targets) aligned in the same direction. Transcription of a gene of interest can be blocked by an "off cassette", which is situated between its promoter and its coding region. This "off-cassette" is flanked by FRT sites and comprises a transcriptional terminator and a marker. As soon as the FLP enzyme is expressed, it binds the FRT sites and excises the "off cassette" through site-specific recombination. As a result, transcription of the gene of interest is enabled, since its promoter and its coding region are no longer interrupted. The spatial control of gene expression is achieved by the usage of two tissue specific promoters driving the FLPase or the gene of interest

4.3) Aim of the project and experimental design

As mentioned above, very few specific downstream targets of the RAS/MAPK pathway and none of the Notch pathway that specify the 1° and 2° cell fates are known. Using plasmids received from Wayne Davis from Erik Jorgensen's lab we planned to achieve cell-lineage specific expression of PAB-1, first in 1° cells and hopefully later in 2° cells. The cell specific expression will be achieved by a combination of two promoters. One promoter driving the FLPase expression in 1° cells as well as other tissues, for example the head. The second construct driving the tagged PAB-1 (GFP::PAB-1), with an "off cassette" in front of it flanked by two FRT sites, with another such promoter that is expressed in 1° cells as well as other tissues, for example the tail. The aim is to have an overlap of expression of the FLPase and the "off cassette"::GFP::PAB-1 *only* in the 1° cells

such that the other tissues, where the two constructs are expressed, will not overlap. Thus, the "off cassette" will be excised and GFP::PAB-1 will be expressed only in the 1° cells. Once this step is achieved, large amounts of worms synchronized to the L3 larval stage, when the cell fates are decided, will be harvested, GFP::PAB-1 bound to different mRNAs will be isolated, and the collected RNA will be analyzed.

4.4) Results

For more detailed results please refer to Debora Kehrli's master thesis

4.4.1) Proof of principle

Davis et. al. used the pharyngeal *myo-2* promoter to drive a construct composed of *FRT::mCherry::FRT::gfp::his-44* and the heat-shock promoter *hsp-16-48* driving expression of the FLPase [4]. We replaced the *myo-2* promoter with the *bar-1* promoter, which is expressed in all VPCs as well as other tissues such as the seam cells and the gut. The FLPase was fused to the *egl-17* promoter, which is expressed in the primary cells at the early stages of vulval development as well as some cells in the head such as the M4 neuron. When expression of mCherry and GFP was examined, a broad expression of mCherry was observed, while GFP was only observed in a few cells, mostly the primary cells (Figure 1).

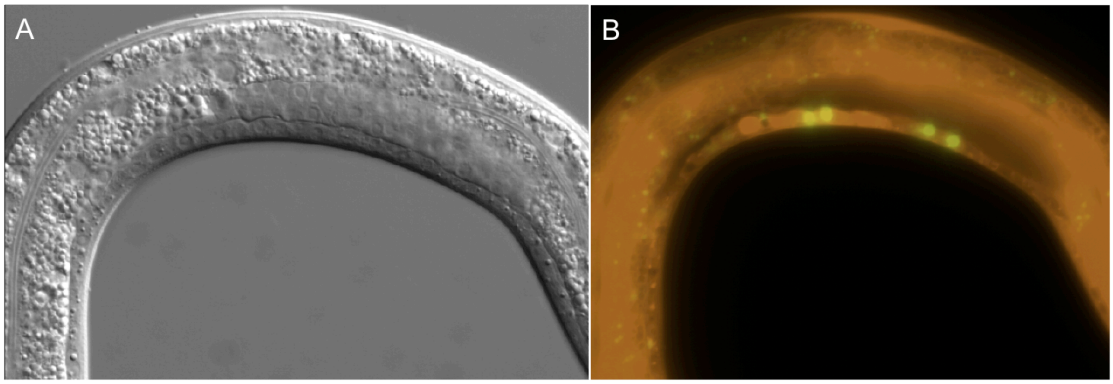

Figure 1. Broad expression of mCherry and specific expression of GFP using the FLPase system. Two constructs, *Pbar-1::FRT::mCherry::FRT::gfp::his-44* and *Pegl-17::FLP* were expressed in N2 animals and expression of the two fluorescent proteins was analyzed using Nomarski (A) and fluorescent (B) microscopy.

4.4.2) Achieving primary specific expression

After a long period of refining the expression pattern by using different combinations of promoters and troubleshooting performed by Debora Kehrli, we followed through using the combination of primers *egl-17* and *vab-23*. *Pegl-17* was used to drive expression of *FRT::mCherry::FRT::gfp::pab-1*, which was integrated using the MosSci technique [6], and *Pvab-23* was used to drive expression of the FLPase, which was injected into the integrated line. We were thus able to get a more specific expression of GFP::PAB-1 in 1° cells (Figure 2A-C), although some non-specific expression still remained (Figure 2D, E).

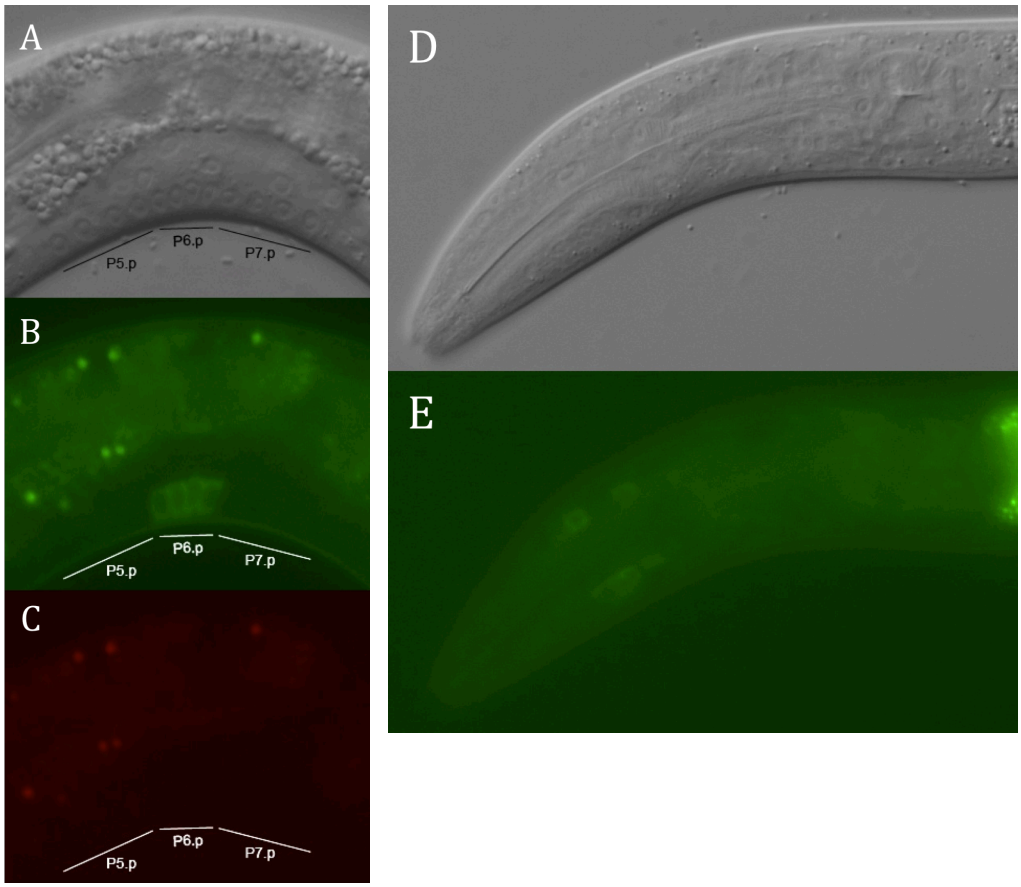

Figure 2. Specific expression of GFP::PAB-1 in 1° cells using the FLPase system. Animals carrying a single integrated copy of *Pegl-17::FRT::mCherry::FRT::gfp::pab-1* were injected with *Pvab-23::FLPase* and expression of mCherry and GFP was examined using Nomarski (A and D) and fluorescence (B, C and E) microscopy. Expression of GFP::PAB-1 can be observed in P6.p (B) with no mCherry expression (C) and in cells in the head (E). Adapted from Debora Kehrli's master thesis.

4.5) Discussion

In this project, we were able to refine a system where expression of genes of interest can be limited to specific cells of interest in a manner not possible before with conventional techniques. This method can be used, as we are currently doing, to perform cell or tissue specific pull down of mRNA using the PAB-1 protein, but one can easily imagine many other ways to use this method. For example, as was recently performed in our lab, Magdalene Adamczyk under the supervision of Stefanie Nusser used this system to express the cell cycle inhibitor *cki-1* in secondary cells in order to arrest the cell cycle and examine its affect on vulval fate specification. But one can also use the system for specific knockdown of genes by expressing in a mutant animal a rescuing construct with its endogenous promoter that contains FRT sites flanking the gene itself and then expressing the FLPase under a specific promoter. For example *let-23(lf)* is a lethal mutation. If one wishes to examine vulval development under such conditions it would be possible to rescue the null mutant with a construct such as *Plet-23::FRT::let-23::FRT* and then express in this worms the FLPase using the *lin-31* promoter. Another usage can be with the heat shock promoter that allows for temporal control of expression, yet is ubiquitously expressed in the worm. Using a combination of a tissue/cell specific promoter and the heat-shock inducible promoter, one can achieve a temporal and spatial control of expression.

In conclusion, the FLPase system can be used in a large range of applications, which will allow researchers to perform experiments that were not technically feasible till now.

4.6) Materials and methods

For materials and methods please refer to Debora Kehrli's master thesis.

4.7) Acknowledgments

I wish to thank members of my group for critical discussion and comments relating to this project, with a very large acknowledgment to Debora Kehrli who performed the majority of the experiments done so far and to Alessandra Buehler, Matthias Morf and Karin Schläpfer who helped me start this project. I am also grateful to the C. elegans genetics centre for providing strains, to Andrew Fire for GFP vectors, to Christian Frøkjær-Jensen and Wayne Davis from Erik Jorgensen's lab for constructs, This work was supported by a grant from the Swiss National Science Foundation to A.H.

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5) Concluding remarks

In this work I have used vulval development as a genetic tool to examine how food quality and the insulin pathway affect RAS/MAPK signaling in the worm. I have been able to show that the nutritional and insulin signals are independent of each other and that both have a significant influence on RAS signaling.

The main components of the insulin pathway are highly conserved between *C. elegans* and other organisms including humans. Many discoveries made with worms hold true for other organisms as well, such as life extension in mice and flies due to reduction in the insulin pathway activity [1, 2]. Furthermore, the insulin pathway has also been found to have a role in cancer in humans (recently reviewed in [3]), yet the detailed molecular mechanism is still not properly understood.

Caloric Restriction (CR) leads to extension of *C. elegans* lifespan and is considered the only current method to extend lifespan in mammals (excluding genetic intervention) [4, 5]. CR has also been shown to affect cancer initiation and progression [6, 7].

CR and the insulin pathway appear to regulate similar processes, and an obvious connection between the two exists as food intake leads to insulin production. However, CR and the insulin pathway also act independently of each other as CR can further enhance lifespan in insulin mutants [8] and insulin mutants have obvious phenotypes not related to the caloric intake of the animal. My results also suggest an independent effect of food on RAS signaling, as the Muv phenotype of animals mutated in the insulin pathway was still enhanced on rich food. Thus CR and the insulin pathway work, at least in part, in parallel also during *C. elegans* vulval development.

But how do the CR and the insulin pathway regulate aging and affect cancer? What is the molecular mechanism, and more importantly, how can we use it to our advantage?

Much of the knowledge regarding the role of the insulin pathway in lifespan and aging originates from research done on *C. elegans,* where our knowledge is relatively well established, with deep understanding regarding the activation, regulation and downstream targets. The FOXO transcription factor DAF-16, the

major downstream target of the insulin pathway, is known to regulate dauer formation and lifespan and many experiments have been performed to describe transcriptional targets of the insulin pathway [9-12]. As more than 35% of *C. elegans* genes have human homologs, it can be interesting to examine how conserved genes modified by DAF-16 are represented in humans.

Research on humans has many limitations. An interesting approach can be to examine expression levels or QTLs (Quantitative Trait Loci) of the different genes of interest. A very long-term follow up is required for this, since humans tend to live longer than worms. Such research would require periodic examination of the tested subject, as evidence suggest that the insulin pathway is active at different levels during different stages of our lives. To slightly simplify the research, data collections can start with older people, for example people that have reached the average lifespan. The research gets another layer of complexity when one takes into consideration the fact that not all tissues age in a similar manner and the tissue specific activity of the insulin pathway. Slowing down aging in one tissue does not mean slowing down aging in general. However, it might be possible to find correlation between insulin signaling and lifespan in humans. Specifically, the downstream targets of the insulin pathway that regulate aging are unknown, and discovering them might help design drugs that modulate specific branches of the pathway to extend healthy lifespan.

The role of the insulin pathway in cancer is well established, yet has so far not been addressed in *C. elegans*. The current work and future experiments should help shed some light on the topic and may better define the molecular and genetic relation between the RAS/MAPK pathway and the insulin pathway.

Research on the topic of caloric restriction appears to be slightly simpler. If modifications of the insulin pathway may have adverse effects in different tissues and require complex external intervention, CR simply requires decreasing the caloric consumption while maintaining the nutritional value. Our body will do the rest. Experiments with *C. elegans*, mice, dogs and even non-human primates have shown that CR can help prevent cancer, help slow down the progression of cancer and extend lifespan.

However, the molecular mechanism of CR is yet unknown. Many believe the insulin pathway has much to do with mediating the effect of CR, yet even so,

further downstream targets are not known. Furthermore, the results from this work suggest a parallel pathway. An interesting starting point for research on the topic can be examining the transcriptional profile of worms under different conditions. For example, *wt* worms under normal conditions, under caloric restriction and when grown on rich food. Further experiments under these conditions can also be performed, such as with different genetic backgrounds, for example *daf-2, age-1* and *daf-16* mutants either alone or, to examine RAS related pathways, in combination with *let-60(gf)* or other mutants in the pathway.

In humans, similar issues regarding the limitations of such research apply as discussed above. However, if in regard to the insulin pathway our question focused more on the output rather than the cause (meaning what is the level and nature of the insulin signal) with the assumption that insulin signaling is the driving force, here the question is different. We would first need to establish that indeed CR can help improve health and lifespan and only later could address the question of how. People who maintain healthy, balanced nutrition, can be compared to people with a less healthy way of life in regard to the chances of developing cancer as well as lifespan. All that needs to be done is collect the data over the years and analyze it. The major problem with such analysis would be the enormous amount of variables to be considered as well as the unknown variables that might obscure the results. However, statistics has its power, and collection of enough data might help.

In conclusion, while research correlating CR and the insulin pathway to lifespan and cancer prevention has shown a prominent connection in model organisms, the complexity of humans, mostly as a free-living organism, i.e. not constrained to the lab but rather exposed to endless environmental and behavioral parameters, makes research on the topic extremely complex. Furthermore, since humans live much longer than model organisms, the follow up on such research will exceed the lifetime of the researcher and of whatever funding the researcher may get. An institution must therefore handle such research with the long-term significance in sight.

Finally, an enormous amount of work, money and resources has been invested over the past decades in search for a cure for cancer. A less attractive option, with much less potential for profit, is to focus on cancer prevention rather than

treatment. Prevention of a disease (e.g. cancer) is much more cost effective than treatment and is of course healthier for the individual. The advantages of CR in model organisms go beyond avoiding overweight. Animals grown on CR live longer, are more active and as mentioned above have smaller chances of developing cancer. Thus, if more focus would go on providing solid proofs for the benefits of CR or other means of cancer prevention, we might finally witness a decline in the number of cancer patients.

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